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By Arthi Kanthasamy

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For the degree of Doctor of Philosophy

Signed by the final examining committee:

David E. Nichols, chair

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This thesis ☐ is ☒ is not to be regarded as confidential. David E. Nichols
Major Professor

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INVESTIGATIONS INTO THE NEUROCHEMICAL MECHANISMS UNDERLYING 3,4-METHYLENEDIOXYMETHAMPHETAMINE (MDMA)-INDUCED NEUROTOXICITY: ASSESSMENT OF THE ROLE OF GABA AND THE DOPAMINE TRANSPORTER

A Thesis

Submitted to the Faculty

of

Purdue University

by

Arthi Kanthasamy

In Partial Fulfillment of the
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of

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LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA	analysis of variance
AS	antisense
B_{max}	the maximum number of binding sites
COX-2	cyclooxygenase-2
DA	dopamine
DAT	dopamine transporter
DOPAC	dihydroxyphenylacetic acid
GABA	γ-aminobutyric acid
GVG	γ-vinyl GABA
5-HIAA	5-hydroxyindoleacetic acid
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine
i.p.	intraperitoneal
K_d	equilibrium dissociation constant
MAO-B	monoamine oxidase-B
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MMAI	5-methoxy-6-methyl-2-aminoindan
MS	missense
ODN	oligodeoxynucleotide
PET	positron emission tomography
ROS	reactive oxygen species
s.c.	subcutaneous
SEM	standard error of the mean
SERT	serotonin transporter
TPH	tryptophan hydroxylase
V_{max}	maximal velocity

ABSTRACT

Kanthasamy, Arthi Ph.D., Purdue University, May 2001. Investigations into the Neurochemical Mechanisms Underlying 3,4-Methylenedioxymethamphetamine (MDMA)-Induced Neurotoxicity: Assessment of the Role of GABA and the Dopamine Transporter. Major Professor: Dr. David E. Nichols.

The current study was undertaken to investigate the mechanism(s) underlying MDMA-induced selective loss of 5-HT neuron terminals in rat brain. We first examined whether enhancement of GABAergic transmission through an irreversible GABA transaminase inhibitor, gamma vinyl GABA (GVG), could counteract MDMA-induced hyperthermia and long-term reductions in 5-HT markers. Pretreatment with GVG (500 mg/kg i.p.) prior to MDMA (40 mg/kg, s.c.) produced significant protection (65-75%) against MDMA-induced loss of 5-HT markers in the cortex, hippocampus, and striatum, and abolished MDMA induced hyperthermia.

In an effort to understand better the neurochemical mechanism underlying these neuroprotective effects of GVG, we studied the role of the GABA-B receptor using the GABA-B agonist, baclofen. Baclofen (18 mg/kg, i.p.) pretreatment blocked both MDMA-induced serotonergic deficits and hyperthermia. These results are consistent with the hypothesis that impaired GABAergic transmission might mediate, at least in part, the neurodegenerative effects of MDMA. Nevertheless, the importance of hypothermia in attenuating the neurotoxic effects of MDMA cannot be excluded.

In the next series of experiments we examined the effect of antisense oligodeoxynucleotide (AS) mediated knockdown of the dopamine transporter (DAT), in order to delineate the role of the DAT in the MDMA neurodegenerative process. DAT-AS unilaterally microinfused into the substantia nigra gave a 70% reduction of the DAT and blocked MDMA-induced neurotoxicity only in the ipsilateral striatum. Interestingly, however, the hyperthermic response elicited by MDMA remained unaltered in the presence of DAT knockdown, suggesting that MDMA-induced hyperthermia may not be obligatory for 5-HT terminal damage. This result suggests that the DAT may play an essential role in MDMA-induced neurotoxicity.

Finally, we briefly examined the role of COX-2 activation in MDMA-induced neurotoxicity. The results indicate a role for COX-2 mediated reactive products in the mechanism(s) of neurodegeneration associated with MDMA, at least in the cortex.

Together, this work demonstrates that multiple neurochemical mechanisms, including the GABAergic system, the DAT, and oxidative stress, may all be involved in MDMA-induced neurotoxicity.

LITERATURE SURVEY

Behavioral Effects of MDMA

3,4-Methylenedioxymethamphetamine (MDMA; figure-1) also known as "ecstasy," is a recreational drug of abuse widely used in dance clubs, small social gatherings, and all-night dancing to technomusic in events known as "raves." Research interests regarding its potential therapeutic benefits were sparked by studies from Shulgin and Nichols in 1978, who reported that the drug induced an "easily controlled altered state of consciousness with emotional and sensual overtones" suggesting that it might be used as an adjunct to psychotherapy. Despite its structural similarity to mescaline and amphetamine the unique ability of MDMA to enhance emotions and empathy without producing sensory disruption and hallucinations led to the classification of the compound in a new category named entactogens (Nichols et al., 1986, 1990). The term entactogen is derived from the Greek roots "en" for within and "gen" to produce or originate, and the Latin root "tactus" for touch. The connotation refers to the drug's ability to promote positive self-assessment and increased self-awareness. Consistent with such effects, MDMA was widely used as an unapproved adjunct to psychotherapy until the mid 1980's.

Concerns over potential neurotoxicity, along with escalating reports of misuse and overdose prompted the U.S. Drug Enforcement Administration (DEA) in 1985, to place

MDMA into Schedule I of the controlled substances act, therefore restricting its availability (Peroutka et al., 1988). Since then, most clinical studies have been based on retrospective anonymous questionnaires. Several retrospective studies relied on the individual's ability to recall past drug effects using an anonymous questionnaire survey in selected groups of individuals with no knowledge of the purity of the drug. For example, a questionnaire study from 1987 performed by Peroutka at Stanford University reported that 39% of young adults among 369 subjects admitted to having used MDMA at least once in the past year. In the last decade, recreational use of MDMA by young people has sharply risen in the United States and Western Europe, most often being ingested during "rave" parties at dance clubs. In a recent epidemiological survey conducted in Switzerland, an estimated 3.5% of 15-34 year-olds had taken at least one recreational dose of MDMA (Giroud et al., 1997). Similarly, in England, a recent report indicates that 4.5 to 6% of 14- and 15-year olds had taken MDMA (Saunders 1995). One major problem encountered in these studies, however, was complete dependence on the subject's ability to recall their exact drug usage history. These studies also lacked a controlled randomized study design, making it difficult to elucidate the pharmacological and toxicological properties in humans. In addition, one cannot be certain of the identity and actual dose(s) of MDMA taken. This problem was circumvented however, by a recently published report (Vollenweider et al., 1998) in drug naïve volunteers that confirmed MDMA's psychopharmacological properties. Due to the fear of long-term adverse consequences and ethical concerns it is unlikely that controlled prospective studies will be performed to elucidate MDMA's effects following repeated use.

Behavioral Effects in Humans

Rats that receive a neurotoxic dosing regimen of MDMA have been shown to develop long lasting reductions in various 5-HT markers, including 5-HT, 5-hydroxyindole acetic acid (5-HIAA), tryptophan hydroxylase, and the serotonin reuptake transporter protein (SERT) (Commins et al., 1987, Molliver et al., 1990, O'Hearn et al., 1988; Schmidt et al., 1987; Ricaurte et al., 1988), which suggest a loss of 5-HT terminal fields. Similarly, in primates, the loss of 5-HT axonal markers was persistent and may, in some regions, be permanent (Fisher et al., 1995; Hatzidimitriou et al., 1999). Preclinical evidence of MDMA-induced neurotoxicity and behavioral deficits suggests that MDMA users might be at risk for the neurotoxic effects of MDMA (McCann et al., 1998).

Few studies are available in the literature concerning MDMA effects in humans. Two prospective studies were conducted in the early 80's to evaluate MDMA's psychotherapeutic effects. In one anecdotal study by Greer and Tolbert (1986), 29 subjects in a clinical setting were provided with one dose of 75-150 mg MDMA followed by an opportunity to receive a "booster" dose of 50-75 mg 2 h later. Most subjects reported that MDMA increased interpersonal communication, improved mood, increased feelings of intimacy, and euphoria, in addition to the symptoms of sympathetic arousal. A day later, some subjects reported negative side effects encompassing mild depression, anxiety, and paranoia. In another study, Downing (1986) assessed the acute effects of MDMA following a single mean oral dose of 165 mg in 21 volunteers who had previous

MDMA experience. Acute effects included positive mood enhancement that included increased closeness to people, euphoria, heightened arousal, heightened sensual awareness, increased emotional and physical energy and decreased appetite. Some volunteers showed gait instability, increased deep tendon reflexes, trismus, and adverse cognitive impairments characterized by difficulty in performing mathematical calculations. About 15% of the subjects participating in the study reported experiencing unpleasant peripheral sympathomimetic effects that included anxiety, ataxia, blurred vision, restlessness, and autonomic hyperarousal. The possibility of bias due to prior knowledge of MDMA's euphoric effects, self-selection criteria, and importance of the mental set between the patient and physician should however, be taken into consideration when interpreting these data.

MDMA's ability to improve mood as described in these anecdotal reports went unsubstantiated due to the lack of a placebo-controlled randomized study design. Recently, however, two studies utilizing placebo-controlled clinical trials have provided preliminary evidence regarding MDMA's acute psychological profile. First, in a recent FDA-approved Phase I study (Grob et al., 1996) evaluating the effects of MDMA, six subjects who had prior experience with MDMA were administered two different dosages (0.25 and 1.0 mg/kg, p.o.) of MDMA utilizing a placebo-controlled double blind, randomized design. The subjects showed no overt physical or psychological discomfort, although a modest increase in heart rate and blood pressure was observed. To what extent previous MDMA use might have impacted these results remains unclear. In a second study (Vollenweider et al., 1998), MDMA was administered at a "recreational" dose of 1.7 mg/kg to 13 drug naïve healthy volunteers in a double blind placebo-

controlled design. Subjects reported an affective state of enhanced positive mood, emotional well-being, little anxiety, moderate depersonalization, and mild derealization effects. In addition, the subjects also experienced moderate hypertension, but the most frequent acute somatic complaints included restless legs, impaired gait, jaw clenching, and lack of appetite. In addition to the acute somatic effects, adverse functional sequelae 24 h following drug ingestion included difficulties in concentrating, insomnia, and brooding. Taken together, these studies suggest that the psychotropic effect of MDMA is different from that produced by classical hallucinogens and stimulants. The authors also point out the potential cardiovascular effects that may be associated with recreational drug use.

In another systematic study by Parrot and Lasky (1998) three groups of young individuals ranging from 19-30 years, which included 15 regular MDMA users who had taken the drug on 10 or more occasions, 15 novice MDMA users who had ingested the drug on fewer than ten previous occasions, and 15 controls who had never taken MDMA but had used other drugs, were assessed for MDMA-induced acute alteration in behavior. All the subjects completed a mood scale battery and cognitive test four times: an initial drug free baseline, at a Saturday night dance club following drug intake, then 2 and 7 days later. Two days following drug ingestion, a pattern of negative mood effects similar to previous studies was reported. In addition, cognitive performance was significantly decreased in MDMA users compared to controls. The data interpretation was hampered however, by methodological problems involving lack of information on past mental history, degree of previous drug use and differences in frequency of drug intake prior to the studies. Taken together, these studies suggest that individuals might be at a potential

risk of developing CNS disorders, such as depression in addition to drug- induced cardiovascular disturbances.

A number of deaths following MDMA use have been reported. Thus, recently much focus has been placed on understanding the relationship between drug use and the occurrence of accidental deaths. There are several reports describing fatal and nonfatal clinical courses related to MDMA use (Henry et al., 1992; Barrett el al., 1993; Brown et al., 1987; Campkin et al., 1992). Clinical manifestations included changes in mental status, restlessness, convulsions, disseminated intravascular coagulation, rhabdomyolysis, hyperthermia, hyperreflexia, and acute renal failure. In a recent study, Coore et al., (1996) reported rhabdomyolysis in addition to pancreatic and hepatic necrosis on post mortem examination of an 18-year old first time MDMA user. Interestingly, among reported deaths there appears to be a lack of correlation between dose ingested and severity of the symptoms. One fatality had a serum MDMA level of 1.26 mg/l, while another patient, whose serum level was 7.0 mg/l, survived the drug overdose. In a recent report (Cohen et al., 1996) a 17-year old Caucasian male died of self-inflicted gun shot injury following his attendance at a rave party in New York. Signs of suicidal behavior, lethargy, and aggressiveness were reported 2 days following his drug use until his suicide the following evening. The authors provide two possible explanations for the drug-induced suicide: 1) MDMA intake might have triggered an acute psychotic episode, 2) alternatively, the individual might have used other drugs in combination with MDMA. The apparent drawback of this case was the lack of availability of his previous medical history, which confounds the identification of the exact cause of his suicide.

The doses used for recreational purposes closely overlap with doses that cause deleterious effects in experimental animal models. Hence, the possibility is raised that long term serotonergic decrements exist in frequent users, despite drug abstinence for a considerable period of time. Technical advances (e.g. PET neuroimaging), in conjunction with improved tests assessing psychobiological effects, might shed some light on long-term effects of repeated drug use. Careful follow up studies of individuals who visit emergency rooms exhibiting signs of acute intoxication may improve our understanding of the putative long-term effects of MDMA.

In summary, the use of MDMA is accompanied by marked behavioral changes including feelings of euphoria, increased energy, and increased desire to socialize that might explain its current popularity as a "rave" drug commonly used in dance clubs where dancing is fast paced and prolonged. Although some of the side effects of MDMA resemble the stimulant effects of amphetamine, MDMA exhibits unique psychotropic effects unlike those elicited by classical psychostimulants or psychedelics. The occurrence of a series of adverse acute mental and behavioral effects, even after a single drug use, questions the perception commonly held among repeat users that MDMA is a benign mood-enhancing drug.

Behavior in Rats

Behavioral studies conducted in animals have confirmed that MDMA exhibits pharmacological effects distinct from classical hallucinogens such as DOM and psychostimulants (e.g. amphetamines) (Oberlender and Nichols., 1988). For example, in

the drug discrimination paradigm LSD only partially substituted and 2,5-dimethoxy-4-methylamphetamine (DOM) failed to provide any substitution in MDMA-trained rats. Conversely, MDMA did not completely substitute in either LSD (Oberlender and Nichols, 1988) or DOM-trained rats (Glennon et al., 1982). Additionally, the enantiomeric selectivity (S>R) of MDMA (Oberlender and Nichols, 1988) is opposite to that seen with phenethylamine psychedelics (R>S) (Glennon and Young, 1984).

MDMA's ability to elicit presynaptic release of 5-HT is thought to be a key component of the unique pharmacology induced by MDMA. In support of this hypothesis was the observation that fluoxetine, a selective 5-HT reuptake inhibitor, attenuated MDMA-induced hyperactivity (Callaway et al., 1990). Qualitative differences between behavioral effects induced by MDMA and those induced by amphetamines have been previously reported. For example, when examining the exploratory and locomotor activity in rats, racemic MDMA (Gold et al., 1989; Gold and Koob 1988) and the more potent S-enantiomer of MDMA (Callaway et al., 1990) exhibited suppression of exploratory behavior, and movement around the periphery of the chamber in unusually straight paths (Gold et al., 1988), whereas amphetamine exhibited varied patterns of activity involving the movement of the animals in a random fashion throughout the chamber. In addition, characteristic differences between locomotor patterns induced by MDMA and hallucinogens were observed. Hallucinogens reduced locomotor activity and investigatory behavior when animals were tested in a novel environment. The behavioral response elicited by hallucinogens was, however, attenuated when the animals were placed in a familiar environment (Adams and Geyer 1985). In contrast, the MDMA-induced increase in locomotor activity was not attenuated when the rats were tested in a

familiar environment (Callaway et al., 1991b), an observation that provides further evidence of psychotropic effects unlike those produced by the classical hallucinogens.

Despite numerous reports demonstrating the unique behavioral effects of MDMA, the receptor mechanism(s) mediating the locomotor enhancing effects of MDMA remain only partially understood. The close resemblance of the behavioral effects of the 5-$HT_{1B/1A}$ agonist RU 24969 and MDMA suggested that activation of the 5-HT_{1B} receptor may be necessary for the locomotor stimulating effects of MDMA. In rats, both drugs were shown to produce increased locomotion, decreased exploratory rearings and hole pokes, and a straight-line pattern of locomotion (Rempel et al., 1993). Furthermore, β-adrenergic antagonists with affinity for 5-HT_{1A} receptors were able to antagonize the MDMA-induced hyperlocomotory reponse (Callaway et al., 1992, Rempel et al., 1993). Taken together, these studies indicate that 5-HT_{1A} receptors might be critical for the mediation of the hyperlocomotory response produced by MDMA.

Recently, Scearce-Levie et al. (1999), using 5-HT_{1B} knockout mice showed that MDMA-induced hyperlocomotion was drastically reduced in KO mice, without significant alteration of the drug induced reduction in exploratory behavior, thereby suggesting that the 5-HT_{1B} receptor might also be partly responsible for the locomotor enhancing effects of MDMA, independent of the MDMA-induced decrease in exploratory behavior. In a parallel study, in order to confirm further the role of 5-HT_{1B} receptors in the locomotor enhancing effects of MDMA, mice were treated with GR 127935, a 5-$HT_{1B/1D}$ receptor antagonist, prior to MDMA. This study found that the mice exhibited an attenuated locomotor response without any significant effect on drug induced suppression of investigatory behavior, similar to knockout mice, hence providing

direct evidence for the role of 5-HT_{1B} receptors in mediating the locomotor enhancing effects of MDMA.

In rats the acute release of 5-HT and subsequent activation of 5-HT receptors are believed to mediate a specific behavioral response known as the "serotonin syndrome" which is elicited at doses higher than 5 mg/kg (Hiramatsu et al., 1989; Spanos and Yamamoto 1989). The syndrome is comprised of low body posture, forepaw treading, head weaving, and tail flicks. Although 5-HT_{1A} receptors have been implicated in the mediation of this behavior (Milan and Colpaert, 1991), the nonspecificity of this syndrome so far precludes any definitive conclusions about the subtype of the receptors involved. Not surprisingly, therefore, MDMA also produces elements of the serotonin syndrome in rats. Furthermore, the S-(+) isomer of MDMA was shown to be more potent in eliciting the 5-HT syndrome, consistent with a role for the serotonin releasing effect of the drug, rather than a direct 5-HT receptor activation, where the R-(-) isomer is known to be more potent. Collectively, these data suggest that drug-induced acute release of presynaptic 5-HT, and subsequent activation of 5-HT_{1A} receptors might be related to the serotonin syndrome elicited by MDMA.

The increase in MDMA use has prompted some researchers to investigate whether the drug possesses significant abuse potential, that is, whether the drug may lead to dependence. The potential for abuse of MDMA has been assessed in experimental animal models, which indicate that the drug possesses only moderate dependence potential. For example, using cocaine substitution studies, non-human primates trained to lever press for intravenous injection of cocaine readily self-administered MDMA, although at a lesser frequency than cocaine (Beardsley et al., 1985; Lamb and Griffith,

1987). Similarly, intracranial self-stimulation studies can be employed to understand the reinforcing effects of psychostimulants. Drugs are examined based on their ability to lower the threshold of electrical stimulation of the mesolimbic system and increased rate of self-stimulation for maintenance of drug seeking behavior. Dose-dependent lowering of the reward threshold was observed up to a dose of 2 mg/kg with MDMA, but LSD lacked this effect (Hubner et al., 1988). Likewise, a study (Lin et al., 1997) comparing the response rates and the threshold frequency for intra accumbal self-stimulation with MDMA, d-amphetamine, and cocaine found that MDMA lowered both the threshold frequency and response rates. Collectively, these data suggest that MDMA shares some of the locomotor enhancing activity and reinforcement properties seen with psychostimulant drugs of abuse such as cocaine and amphetamine.

Conditioned place preference (CPP) is another paradigm considered to be a measure of drug reinforcing behavior. An increased CPP response in rats may indicate increased vulnerability to the rewarding effects of drugs of abuse and subsequently increased dependence potential. Recently, Marona-Lewicka et al. (1996) examined the rewarding and aversive effects of MDMA and other substituted amphetamine analogs. MDMA was shown to produce a maximal effect in the CPP test at doses of 5 and 10 mg/kg. The α-ethyl homologue of MDMA (MBDB) however, was found to produce maximal effect only at 10 mg/kg and was at least 2.5-fold weaker when compared to MDMA. Microdialysis studies in the nucleus accumbens revealed that MDMA produced pronounced increases in extracellular levels of DA and its metabolite DOPAC, whereas MBDB was without any significant effect on dopaminergic parameters, thus implicating the dopaminergic system in the rewarding properties of MDMA. In another study

(Ashby et al., 1999) rats chronically treated with MDMA at a dose of 20 mg/kg, b.i.d, for 4 days showed a significantly higher CPP response to cocaine 2 weeks after the last dose of MDMA. These results suggest that prior exposure to MDMA might produce sensitization to cocaine, potentially enhancing its abuse potential. Extrapolating the present study to humans raises the possibility that MDMA abusers may be at greater risk for cocaine dependence than previously thought.

In summary, the unique behavioral and locomotor stimulating effects of MDMA support its classification as a novel pharmacological agent. In addition, behavioral studies conducted in rats implicate the involvement of presynaptic 5-HT release, and 5-HT_1 receptor activation, in particular the 5-HT_{1A} and 5-HT_{1B} receptors, in the behavioral effects mediated by MDMA. Taken together, these data support the hypothesis that despite structural similarity to other phenyalkylamines and hallucinogens, MDMA exhibits a unique pharmacological profile that might possess the potential for novel patterns of abuse among humans.

Short-Term Biochemical Actions

In the last decade intense scrutiny of the acute effects of MDMA has improved our understanding of the neurochemical and behavioral correlates of the effects of this drug on monoaminergic transmission. There is strong evidence that MDMA's pharmacological effects are attributed to its monoamine releasing capability (figure 2). Specifically, potent MDMA-induced release of 5-HT together with less efficacious DA release appear to be critical determinants in the induction of MDMA-induced behavior,

as well as selective serotonergic terminal denervation, and acute psychostimulatory effects. Acute treatment with MDMA also produces blockade of monoamine uptake, inactivation of tryptophan hydroxylase, depletion of intraneuronal stores of 5-HT and its metabolite 5-HIAA, and inhibition of monoamine oxidase. Moreover, there is compelling evidence that MDMA's behavioral effects might be also partially attributed to indirect activation of the 5-HT_{2A} receptor. In the following section the short term biochemical consequence of MDMA will be discussed in relation to its receptor affinity profile, release and uptake inhibition of monoamines, inhibition of tryptophan hydroxylase, and modulation of monoamine levels.

Receptor Affinity Profile

MDMA has moderate (low micromolar) affinity for most of the neurotransmitter receptors evaluated thus far. For example, previous studies have shown that MDMA has micromolar or greater K_i values for the adrenergic, dopaminergic, cholinergic, and opioid receptor subtypes (K_i values < 6 μM) (Battaglia et al., 1988a; Pierce and Peroutka 1988). In addition, MDMA also possesses low affinity for the 5-HT_1 receptor family. Ligand binding studies using [^{3}H] ketanserin displacement revealed that the R-(-) enantiomer of MDMA was 4-5 times more potent than the S-(+) isomer (Lyon et al., 1986). These results demonstrate the stereoselectivity of MDMA for binding to 5-HT_2 receptors. In another study, racemic MDMA displaced [^{3}H] ketanserin with a K_i of 8.3 μM, compared with the displacement of [^{3}H]-DOB which was 214 nM. The higher affinity for the DOB binding site might reflect agonist activity of the drug at the 5-HT_2 receptor. Interestingly,

the S-(+) stereoisomer of MDMA was found to be more active in humans (Shulgin et al., 1978), hence arguing against a role for direct receptor activation in the behavioral effects of MDMA.

It was initially reported that [^{3}H]-MDMA binds to rat cortical membrane homogenates with a K_D of approximately 100 nM and a B_{max} of approximately 30 fmol/mg of protein (Battaglia et al., 1988). Furthermore, p-chloroamphetamine (PCA) and methamphetamine were the only compounds shown to displace [^{3}H]-MDMA with moderate affinity. Interestingly, however, this evidence was disputed by another study (Wang et al., 1987), which reported that this binding was actually to the glass fiber filters. Elimination of specifically bound [^{3}H]-MDMA using glass fiber filters presoaked in 0.3% polyethylenimine (PEI) showed that the previous report did actually represent artifactual binding. In a later ex vivo study it was demonstrated that MDMA has appreciable affinity for monoaminergic uptake sites. Zaczek et al. (1990), using a centrifugation technique showed that [^{3}H]-MDMA was incorporated into synaptosomes and labeled binding sites with affinities in the high nanomolar to lower micromolar range. In addition, the binding was blocked by paroxetine and desipramine whereas mazindol was less efficacious, indicating that the radiolabelled MDMA binds competitively to selective sites in the synaptosomes, presumably via interaction with the 5-HT uptake carrier. Subsequently, the existence of a second site dependent on the concentration of sucrose was revealed. To date the relative importance of potential high and low affinity sites labeled by [^{3}H]-MDMA remains unresolved.

Battaglia et al. (1988), using receptor binding analysis, have determined the potency of MDMA for the monoamine uptake sites and reported K_i values of 0.6 μM for

the SERT (serotonin transporter sites), 15.8 μM for the NET (norepinephrine transporter sites) and 24.4 μM for the DAT (dopamine transporter sites). The study illustrates the 40-fold higher potency of MDMA for the SERT in comparison to the catecholamine uptake sites, consistent with MDMA's selective serotonergic properties.

In summary, MDMA has marginal affinity for most of the monoamine receptors assessed so far, with higher affinity for 5-HT uptake sites.

Uptake Inhibition and Release of Monoamines

The biogenic amine transporters are primarily responsible for terminating the actions of monoamines and subsequently limiting the postsynaptic effects of the released monoamines. The effects of MDMA depend on the ability of MDMA to interact with the biogenic amine transporters, leading to monoamine release, thereby resulting in increased synaptic concentrations of monoamines. Two particular, molecular sites of action have been identified in the drug-induced modulation of monoaminergic transmission, namely the plasma membrane transporter and the vesicular membrane transporter (VMAT-2). The existence of a carrier-mediated mechanism in facilitating monoamine release is a well-established phenomenon whereby the ability of MDMA to interact with the transporter or block uptake, or both might be crucial for induction of the neurotoxic cascade that follows a high dose of MDMA. In this context, recent studies using platelet membrane preparations have demonstrated that MDMA acts as a substrate at the SERT and subsequently induces efflux of 5-HT via an exchange-diffusion mechanism mediated

by the transporter (Rudnick and Wall 1992). Thus, in this section studies supporting a role for monoamine transporters in the effects of MDMA will be discussed in detail.

The potent 5-HT releasing effects (Nichols et al., 1982; Nash et al., 1994) and inhibition of reuptake (Steele et al., 1987; Johnson et al., 1986) resulting in enhanced serotonergic transmission are thought to underlie MDMA's unique behavioral actions and to be essential for long-term neurotoxic effects. Several laboratories using a variety of neurochemical techniques have supported this hypothesis. For example, MDMA's potent 5-HT releasing effect has been demonstrated using synaptosomes (Nichols et al., 1982), superfused brain slices (Johnson et al., 1986; Schmidt et al., 1987), cultured neurons (Gu and Azmitia, 1993) and also in vivo microdialysis studies (Brodkin et al., 1993; Gudelsky and Nash, 1996). In vitro studies indicate that MDMA is a more potent releaser of 5-HT than DA. Steele et al. (1987), using striatal slice preparations, demonstrated that MDMA was 10-fold more potent in inducing 5-HT release in comparison to DA. A large number of in vitro studies support the hypothesis that MDMA interacts with the SERT by acting as a substrate at the transporter protein. Indeed, Rudnick and Wall (1992) proposed a model whereby MDMA was postulated to be taken up into the 5-HT terminal via the SERT. Once inside, MDMA competitively binds to the vesicular membrane transporter (VMAT) and redistributes the vesicular contents to the cytoplasm via dissipation of the transmembrane pH gradient. Consequently, the increased cytoplasmic concentration of 5-HT was presumed to provide the concentration gradient necessary for reverse transport of the 5-HT out of the terminal by the SERT. The involvement of a carrier-mediated mechanism in MDMA-induced 5-HT release was further supported by Gudelsky and Nash (1996) using microdialysis studies, who demonstrated that in the striatum of freely

moving rats MDMA-induced a concentration dependent release of 5-HT that was attenuated by fluoxetine pretreatement. Although MDMA has high affinity for the monoamine uptake carrier, because of the lipophilic characteristic of the drug a passive diffusion across the synaptic terminal membranes cannot be excluded as a contributing factor.

In addition to the 5-HT releasing effect of MDMA there is substantial evidence from both in vivo and in vitro studies to show increased DA efflux following acute MDMA exposure. For example, MDMA and its structurally related analogs have been shown to mediate a concentration dependent release of [^{3}H]-DA from striatal slices (Johnson et al., 1986; Schmidt et al., 1987). Additionally, MDMA also inhibits uptake of [^{3}H]-DA into striatal synaptosomes (Steele et al., 1987). The increase in the extracellular concentration of striatal DA produced by systemically administered MDMA could be attenuated by DAT inhibitors such as GBR 12909 (Nash and Brodkin, 1991), and mazindol (Shankaran et al., 1999), hence suggesting that a carrier-mediated mechanism might play a key role in the pronounced in vivo increase of extracellular DA following MDMA.

The dependence on specific stereochemical requirements to facilitate DA release has been closely examined. Increasing the length of the N-alkyl substituent was without effect on the serotonergic potency of the drug, however, lengthening of the N-alkyl group from methyl (MDMA) to ethyl (MDE) produced a significant reduction in [^{3}H]-DA releasing capacity from striatal slices (Schmidt et al., 1987). The aforementioned studies were validated using in vivo microdialysis studies, confirming the importance of N-alkyl substituents (Nash and Nichols, 1991). The stereoselectivity of isomers of MDMA to

elicit DA release has also been studied; the S-(+) isomer was found to be a more potent releaser of DA than the R-(-) isomer both in vitro (Johnson et al., 1986) and in vivo (Hiramatsu and Cho, 1990).

MDMA also has been shown to induce release of NE from hippocampal slices (Fitzerald and Reid, 1990). The underlying assumption that MDMA binds to the norepinephrine transporter to mediate NE release was based on studies demonstrating that MDMA-induced release of NE was blocked by desmethylimipramine, a norepinephrine transporter inhibitor. As is the case for DA release, the S-enantiomer is substantially more potent, while the R isomer possessed diminished ability to elicit NE release from brain slices (Steele et al., 1987). Therefore, a calcium independent, carrier-mediated mechanism might be an essential component in producing the release of monoamines.

In the last decade several in vivo and in vitro studies have established that the non-vesicular releasing properties of MDMA are among the most predominant effects that underlie the drug's unique psychotropic effects. MDMA particularly appears to exert potent effects on 5-HT release in comparison to its catecholamine releasing properties. Also, MDMA appears to exhibit stereochemical selectivity in terms of its releasing properties, whereby the S isomer is more potent than the R isomer, which is particularly evident in relation to its DA releasing effects. Based on the fact that MDMA has modest affinity for most of the neurotransmitter receptors studied, together with the dependence on a carrier mediated mechanism in facilitating release of monoamines, a detailed analysis of MDMA-induced 5-HT release might improve our understanding of the underlying mechanism responsible for selective 5-HT neurotoxicity.

Inhibition of Tryptophan Hydroxylase

Tryptophan hydroxylase (TPH) is the initial and rate limiting enzyme in the 5-HT biosynthetic pathway. Chronic exposure or a single high dose of MDMA appears to modulate TPH activity, thereby resulting in diminished serotonin levels. Indeed, studies have shown that reduction of TPH activity precedes the persistent depletion of 5-HT and 5-HIAA that follows MDMA exposure (Schmidt et al., 1986; Stone et al., 1987a, 1987b; Schmidt and Taylor, 1988). The mechanism of TPH inactivation was shown to involve a reduction in the V_{max} without an apparent change in the K_m for the substrate. Similar to the stereoselective effects of MDMA on 5-HT release, enantiomeric specificity of MDMA in attenuating the catalytic effects of TPH has been shown, whereby the S-enantiomer produced more potent attenuation of TPH activity than its antipode the R-isomer. The TPH activity remained lowered even at 24 h, although 5-HT and 5-HIAA had returned to normal levels following a neurotoxic regimen of MDMA. Therefore, the degree of recovery of 5-HT levels appears to be independent of changes in TPH activity, at least within the first 24 h. In addition, several investigators have demonstrated the importance of dosing regimen and exposure time for influencing the loss of TPH activity. For example, a bolus injection of MDMA into the rat brain failed to affect TPH, whereas continuous infusion of the same dose produced significant reductions in TPH activity (Schmidt and Taylor, 1988).

The exact mechanism of MDMA-induced TPH inactivation remains poorly understood. Several hypotheses have been advanced, however, in an attempt to unravel

the mechanism. One proposal is that drug-induced presynaptic release of 5-HT acts through a feedback or autoreceptor mechanism, thereby producing downregulation of TPH activity. The requirement of 5-HT release for decreasing TPH activity was illustrated by studies showing that pretreatment with 5-HT uptake inhibitors such as fluoxetine not only afforded protection against the persistent loss of 5-HT markers, but also blocked the acute loss of TPH activity (Schmidt and Taylor 1987, Schmidt and Taylor 1990). Moreover, in another study, ketanserin, a $5\text{-}HT_{2A/2C}$ antagonist, and methiothepin, a 5-HT autoreceptor antagonist, were shown to block TPH inactivation, even when administered 3h after MDMA. These studies also showed that there is a time frame within which TPH catalytic activity can be restored despite exposure to a neurotoxic MDMA dose.

Another putative mechanism for TPH inactivation involves oxidative stress. According to this hypothesis, generation of reactive oxygen species (ROS) via oxidation of DA or metabolic degradation of MDMA might play an important role in TPH inactivation. Stone et al. (1989) showed that MDMA-induced inactivation of TPH in rat cortical homogenates can be completely reversed by incubation of the homogenates in an oxygen free atmosphere for 20-24 h or in the presence of DTT 3h, but not 18 h after drug treatment. The authors hypothesized that the susceptibility of sulfhydryl groups in TPH to oxidation by ROS might explain the loss of activity. This idea was supported by a recent finding (Kuhn et al., 1999) whereby exposure of TPH to DA under mild oxidizing conditions (iron and H_2O_2) resulted in a concentration-dependent inactivation of the enzyme. The authors also demonstrated the formation of DA quinones that covalently modified cysteinyl residues in TPH, hence resulting in the loss of TPH catalytic activity.

The authors hypothesized that TPH could be a target for DA quinones in vivo after a neurotoxic dose of substituted amphetamines such as MDMA. Collectively, these results provide further support for a role of ROS in the MDMA-induced loss of TPH activity.

In conclusion, at least three possible scenarios appear to be involved in the MDMA-induced loss of TPH activity: 1) autoreceptor-mediated downregulation of TPH; 2) oxidative stress-mediated oxidation of sulfhydryl groups of TPH; 3) formation of a toxic metabolite of MDMA, possibly a quinone that covalently reacts with TPH. Regardless of the mechanism of TPH inactivation, several studies have suggested that MDMA-induced inactivation of TPH accompanies the persistent 5-HT axon loss and can also serve as a marker for 5-HT terminal degeneration.

Modulation of Monoamines

Among the most prominent acute actions of MDMA is the production of dramatic increases in forebrain tissue levels of monoamines, especially in regions that receive 5-HT innervation. Numerous studies have examined the dose, frequency, and route of administration essential to produce neurotoxicity. For example, Ricaurte et al. (1987) showed that a single dose of 10 mg/kg produced long lasting decreases in serotonergic markers in forebrain regions of rats. Similarly, rats that were administered a single dose of 40 mg/kg were shown to have long lasting reductions in 5-HT, 5-HIAA, and 5-HT uptake sites 2 weeks after drug administration (Johnson et al., 1992). The total dose necessary to produce neurotoxic effects over a four-day period is typically 20-80 mg/kg in rodent models (Fischer et al., 1995, Insel et al., 1989). However, many

researchers have used a dose of 20 mg/kg twice daily for four days to produce robust effects on drug-induced long-term reductions in 5-HT markers. Furthermore, the metabolic clearance in rodents is much higher than in human and thus humans might be sensitive to the neurodegenerative effects of MDMA at much lower doses. Indeed, non-human primates have been shown to be more sensitive to MDMA than rodents. Taken together, these data suggest that doses, dosing regimen, and species difference might be important factors that influence the expression of neurotoxicity.

Schmidt et al. (1987) first demonstrated that a single dose of MDMA produced a biphasic response in relation to the acute depletion of 5-HT. The first phase involves a rapid depletion of 5-HT levels that lasts 3-6 h after drug administration, followed by a variable recovery that is evident by 24 h. Subsequently, the second phase occurs 3-7 days following drug administration, during which detrimental effects on the 5-HT neurons that contribute to loss of terminal integrity become evident. The parallelism in the time course of 5-HT, 5-HIAA, and TPH changes suggests that perhaps inhibition of TPH might contribute to the acute decreases of 5-HT and 5-HIAA. The evidence that selective serotonin reuptake inhibitors (SSRIs) such as citalopram and fluoxetine can block both MDMA-induced acute 5-HT release (Gudelsky and Nash 1996) as well as long term depletion (Schmidt et al., 1987) suggests the involvement of a 5-HT carrier mediated process. Interestingly, despite complete protection against the long-term neurodegenerative effects of MDMA, fluoxetine was able to provide only partial protection against MDMA-induced inactivation of TPH. Thus, TPH inactivation may simply be a marker of intracellular stress, rather than a contributor to the neurotoxic effects of MDMA.

Regional brain differences in the sensitivity to MDMA-induced acute alterations in monoamine levels have also been reported. In rats the hippocampus and cortex displayed 20-50% reductions in 5-HT and 5-HIAA levels following a single dose of 10-20 mg/kg acutely, however, the diminished levels showed signs of recovery within the next 24 h. In contrast, striatal levels of 5-HT and 5-HIAA showed marked reductions with increasing time (Schmidt et al., 1986; McNamara et al., 1995). In contrast to the long-term serotonin depleting effects of MDMA, only slight alteration of dopaminergic markers was observed following MDMA administration (Schmidt et al., 1987; Colado et al., 1999d). Hence, depending on the local environment, different regions apparently have differential sensitivity to the long-term effects of MDMA.

The requirements for 5-HT release, behavioral effects, TPH effects, and MDMA-induced DA release show similar steroselectivity. The steroselectivity for inducing long-term neurodegenerative effects in vivo (S > R) is the same as for in vitro monoamine release (Johnson et al., 1986, 1987; Schmidt et al., 1987).

Dose-dependent effects of MDMA on the magnitude of acute monoamine release have been consistently observed using various techniques. For example, a dose of 10 mg/kg MDMA produced a greater and more rapid release of DA in the striatum than in the nucleus accumbens as determined by in vivo voltametry (Yamamoto and Spanos, 1988). In addition, using microdialysis studies, a dose-dependent increase in the magnitude of MDMA-induced DA and 5-HT release in the striatum and cortex have been consistently observed (Gudelsky et al., 1995; Yamamoto et al., 1995; Shankaran et al., 1999). The occurrence of monoamine release in regions in which serotonergic and dopaminergic neurons are in close synaptic apposition is consistent with a functional

interaction between DA and 5-HT in mediating the acute as well as long term actions of MDMA. In support of this hypothesis, pretreatment with agents such as fluoxetine, a 5-HT uptake inhibitor (Gudelsky and Nash, 1995), MDL-100,907, a $5HT_2$ receptor antagonist (Schmidt et al., 1992), and mazindol, a DAT inhibitor, attenuated acute release of 5-HT in conjunction with decreased DA release.

In summary, the release of monoamines and the resulting dramatic changes in monoamine levels are probably mediated by the interaction of MDMA with the monoamine carriers. The excessive release of DA, and 5-HT, with the subsequent activation of $5\text{-}HT_{2A}$ receptors also appear to be critically involved in MDMA-induced behavioral and neurotoxic effects.

Long-Term Neurotoxic Effects

Previous studies have shown that the initial release of 5-HT ultimately leads to a massive depletion of serotonin during the first few hours after MDMA administration, with a second phase during which normalization of monoamine levels occurs at 24 h post drug administration. A third phase occurs with the irreversible loss of 5-HT markers that is believed to last from about three days until at least several months after drug administration. The biochemical manifestations of this secondary long-term reduction of 5-HT markers are characterized by: dramatic reductions in 5-HT, 5-HIAA, TPH activity, and density of the SERT (Battaglia et al., 1988; Zhou et al., 1996). Additionally, immunohistological studies have shown a severe loss of 5-HT terminal morphology, comprised of swollen 5-HT presynatic varicosities, fragmentation, and dilation of 5-HT

axons (O'Hearn et al., 1988; Molliver et al., 1990). In the following section the possible clinical manifestations of the neurodegenerative changes induced by MDMA will be discussed in detail.

Reductions in 5-HT, 5-HIAA, SERT, and TPH

Despite a large body of evidence supporting the loss of 5-HT terminals, the exact mechanisms by which MDMA causes the degeneration of 5-HT nerve terminals and axons remain poorly understood. One fact that is generally agreed upon is that a single neurotoxic dose or repeated doses of MDMA in rats produce persistent decreases in regional brain 5-HT, 5-HIAA, and SERT density (Commins et al , 1987; Battaglia et al., 1987; 1988; Ricaurte et al., 1988; Schmidt et al., 1986; 1987). Nevertheless, dopamine levels do not show depletions in comparison to 5-HT. This suggests that serotonergic and dopaminergic parameters are differentially vulnerable to the neurotoxic effects of MDMA, the former being less susceptible than the latter.

As mentioned previously, MDMA-induced long term reduction in TPH was demonstrated by Stone et al. (1989a), who showed that TPH inactivation follows a distinct time course, whereby complete reversal of TPH inactivation is possible within 3h of MDMA exposure, but that irreversible activation of TPH occurs 6h following MDMA. The mechanism underlying enzyme inactivation is attributed to reduced V_{max} rather than altered substrate affinity (Schmidt et al., 1987a). In another study, Schmidt et al. (1990a) reported that the compromise of the redox status within the 5-HT neuron might be responsible for the persistent loss of TPH induced by MDMA. The ability of an

antioxidant such as ascorbic acid or GSH to protect TPH from irreversible inactivation suggests a role for oxidative stress in the neurotoxic effects of MDMA. Furthermore, the hypothesis that MDMA-induced formation of ROS might result in the oxidation of one or more sulfhydryl groups was tested by Kuhn and Arthur (1998) who showed that dopamine quinones produce covalent modification of cysteine residues in TPH, hence providing evidence that perhaps redox recycling of a TPH-quinoprotein could be an intermediate in the neurotoxicity process. Taken together, these data support the hypothesis that MDMA-induced formation of ROS might overwhelm or exhaust the antioxidant machinery within the 5-HT neuron, which ultimately results in the selective loss of 5-HT terminals.

Persistent reductions in SERT have been demonstrated using radioligand binding studies, synaptosomal uptake studies, autoradiography studies, and immunohistochemical studies. For example using [^{3}H]-paroxetine binding, MDMA has been reported to produce decreases in the density of SERT with no significant alteration of K_D (Battaglia et al., 1987; Johnson and Nichols 1989; Nichols et al., 1990). In another study conducted in rats, Seiden and Sabol (1996) administered MDMA at a dose of 20 mg/kg twice daily for 4 days. Neurochemical analysis performed at 2, 8, 16, 32 and 52 weeks after drug administration indicated that [^{125}I]-RTI-55 binding to the DAT in striatal homogenates showed no significant alteration at any time point assessed, however, a significant reduction in the density of SERT was observed in the hippocampal and the frontal-parietal homogenates 2-16 weeks following drug. Nevertheless, a full recovery of SERT was observed in the hippocampus and cortex by the end of a 52 wk period. The occurrence of a rostral-caudal gradient SERT recovery process, in both hippocampus and

cerebral cortex, confirms previous reports of tissue specific effects of MDMA on 5-HT neurons.

Histological Changes

Studies conducted in rats have consistently shown that repeated MDMA exposure leads to dramatic long-term reductions in the activity of TPH, brain concentrations of 5-HT, and 5-HIAA, and reductions in the density of the SERT. These changes could be the result of a simple downregulation of 5-HT markers and function in response to a high dose of MDMA. Thus, in order to rule out this possibility, correlative anatomical studies are of immense help in showing that the deficits of 5-HT axon terminal markers in MDMA treated rats are indeed related to the actual loss of 5-HT terminal integrity.

Several types of histological studies can be utilized to confirm serotonin terminal denervation: 1) immunocytochemical studies of the cell bodies to reveal the cell number and the metabolic synthesis capacity of the neuron. 2) Nissl-staining provides information regarding pathological changes associated with neurodegenerative effects namely, chromatolysis. 3) silver staining has been successfully used to demonstrate cell death, however, the technique is subject to variability depending on appropriate survival times. Hence, careful interpretation of the data is warranted.

Subsequent to biochemical studies revealing persistent loss of 5-HT markers, monoamine histofluorescence methodology was used to correlate decreased immunofluorescence with decreased 5-HT levels. For example, the loss of 5-HT axon density has been assessed by using an antibody to 5-HT. These immunohistochemistry

studies revealed dramatic reductions in 5-HT axon density in the forebrain regions two weeks after MDMA administration (O Hearn et al., 1988). In addition, silver staining showed a greater degree of degeneration in striatal and cortical axonal projection areas (Commins et al., 1987) following parenteral or oral administration of a large dose of MDMA. Similar results have been reported in nonhuman primates, although the doses that produced the neurotoxic effects were much lower when compared to rodents (Ricaurte et al., 1988). Collectively, the histochemical data mirror biochemical studies confirming that the neurotoxic effects of MDMA are limited to the 5-HT terminal projection fields, including the hippocampus, striatum, and cortex, in both rodents and non-human primates.

Morphological studies demonstrate that a subpopulation of the 5-HT immunoreactive axons, which includes the fine fibers with small fusiform terminal varicosities, are particularly vulnerable to the persistent neurodegenerative effects of MDMA (Battaglia et al., 1988). Indeed, Molliver et al. (1990) showed that MDMA produces degeneration of the fine fibers while leaving the larger beaded terminals intact. The manifestations of degeneration initially included swollen abnormal looking forms and, one to two weeks later, loss of 5-HT terminals was observed. Consistent with previous biochemical studies, a pernicious effect on the 5-HT neurons, but an absence of any morphological alterations in catecholamine axons in rats and monkeys was observed.

There is compelling evidence that MDMA selectively targets 5-HT axon terminals. Furthermore, regional differences in the magnitude and extent of neuronal injury and subsequent rates of recovery following a neurotoxic dose of MDMA have

been reported. For example, Lew et al. (1996) showed that the rate of recovery varies according to the brain region and that recovery of 5-HT markers in the neocortex and hippocampus followed a rostral-caudal gradient. The existence of tissue specificity in the neurotoxic response was demonstrated in studies that showed profound reductions in the cortex and hippocampus, minimal reductions in the hypothalamus and striatum, and absence of any deleterious effects in other regions that receive 5-HT innervation (Ricaurte et al., 1985; Schmidt et al., 1987; Stone et al., 1986).

In the past few years considerable interest has been focused on understanding the 5-HT reinnervation pattern following MDMA-induced neuronal injury. The major question that was addressed in several studies was the reorganization pattern following the "pruning" of 5-HT neurons. Fisher et al. (1995), using squirrel monkeys, demonstrated that axonal sprouting after MDMA may be region dependent. An abnormal reinnervation pattern was observed in monkeys, whereby the distal targets (dorsal neocortex) remained denervated 18 months after MDMA administration, while some proximal regions (hypothalamus, amygdala) were hyperinnervated. In another study (Hatzidimitriou et al., 1999) performed 7 years after MDMA administration, abnormal 5-HT innervation patterns were still evident in treated monkeys, although the extent of injury was somewhat less severe than at the 18-month period. The authors concluded that the recovery time and the magnitude of recovery following MDMA exposure are influenced by a variety of factors that include the dose, dosing regimen, brain regions examined, species, proximity of the terminal to the cell body and myelinated fiber tracts.

The lack of availability of suitable neuroimaging techniques has, until recently, hindered the direct visualization of MDMA-induced alterations in living brains. In a recent report (Scheffel et al., 1998) [^{11}C]-(+)-McN5652, a ligand selective for 5-HT transporters, was used to assess the status of 5-HT neurons in the living baboon brain. PET studies at 13, 19, and 40 days post-MDMA (5 mg/kg, s.c.) revealed marked reductions in SERT. Reductions ranged from 44% in pons to 89% in the occipital cortex. Nine and thirteen months following drug administration a persistent reduction was observed in the neocortex, whereas hypothalamus showed increases in the apparent recovery of SERT. Measurement of 5-HT axonal concentrations following sacrifice of the animals correlated well with regional SERT levels imaged with PET studies. The results are consistent with the hypothesis that MDMA is also a potent 5-HT neurotoxin in nonhuman species. A PET tracer namely [^{11}C]-(+)-McN5652 was used in characterizing the neurotoxic effects of MDMA in human MDMA users (McCann et al., 1998). This study investigated the status of brain 5-HT neurons in 14 previous MDMA users who were abstaining from drug use at the time of the study and 15 controls who had never used MDMA. The data showed global as well regional reductions in SERT binding compared with controls. Additionally, a positive correlation between decreases in SERT binding and extent of previous MDMA use was also observed. Taken together, PET neuroimaging studies provide direct evidence of a decrease in SERT levels in human MDMA users.

In summary, acute exposure to MDMA produces dose-dependent reductions in 5-HT, 5-HIAA, the density of SERT, and TPH activity. Although MDMA produces marked acute release of monoamines, only the serotonergic system is selectively

damaged, whereas no long-term alterations in dopaminergic markers occur. It is worth noting again the enantiomeric selectivity exhibited by MDMA where the S-isomer is the more active isomer in humans and animals, in eliciting psychotropic effects in the former and inducing serotonergic deficits in the latter. Despite the controversy over generalizability of animal studies to humans (Holland et al., 1999), the doses of MDMA that produce neurotoxic effects in animals closely overlap with the range of those used by humans (Ricaurte et al., 2000). Thus, findings in different species raise great concern that human MDMA users are at a potential risk of incurring irreversible 5-HT neuronal injury. The increased vulnerability of nonhuman primates suggests that repeated MDMA users might be at a risk of incurring MDMA-related 5-HT neural injury and, possible functional sequelae. Indeed, the availability of advanced PET technology has provided direct evidence of a decrease in SERT in human MDMA users.

The Role of Dopamine in Neurotoxicity

There is strong evidence that DA plays an essential role in the MDMA-induced degeneration of 5-HT terminals. Pharmacological interventions with agents that interfere with dopaminergic transmission have been shown to be effective in attenuating the neurotoxic effects of MDMA. Although the exact mechanism underlying DA mediated neurodegeneration is poorly understood, several studies have reported evidence to confirm that DA is an important contributor to the serotonergic neurotoxicity process. For example, Nash and Brodkin (1991) using microdialysis studies showed that MDMA infused into the anterolateral striatum produced

concentration and time-dependent release of DA. However, peripheral administration of a DAT inhibitor, GBR-12909 or mazindol, produced significant attenuation of MDMA-induced DA release as well as attenuation of long-term serotonergic deficits. The existence of a positive correlation between the ability to elicit a neurotoxic response and relative ability to increase extracellular DA release has been previously reported using both in vivo (Nash and Nichols, 1991) and in vitro studies (Johnson et al., 1986). Furthermore, depleting neuronal DA stores with alpha-methyl-para-tyrosine, a DA synthesis inhibitor, attenuated the persistent 5-HT deficits induced by MDMA in the striatum and cortex (Stone et al., 1988; Schmidt et al., 1990). In a similar manner, depletion of vesicular catecholamine stores with reserpine inhibited the persistent reductions of 5-HT axonal markers induced by MDMA (Schmidt et al., 1990). Likewise, bilateral lesioning of the nigrostriatal dopaminergic system blocked the long-term neurotoxic effects of MDMA (Schmidt et al., 1990). In a reciprocal fashion, administration of L-dopa, a precursor of DA, was found to exacerbate neurotoxicity (Schmidt et al., 1990). Recently, the importance of DA in the neurotoxicity process was indirectly demonstrated in rat pups. In rats a fully functional DA system is not in place until about PND 21. Thus, a single dose of MDMA (20 mg/kg s.c.) administered at different postnatal stages (PND 2, 14, 21, and 35) produced long-term reductions in 5-HT levels in all the brain regions examined only at PND 35, although hyperthermia was observed in all the pups. Conversely, MDMA administered in combination with L-DOPA produced lasting reductions in 5-HT and SERT levels in the hippocampus and in the frontal cortex of PND 21 rats. In a similar fashion adult rats that were previously lesioned with 6-OH DA were resistant to the neurotoxic effects of MDMA, however,

coadministration of L-dopa and MDMA was found to reinstate the neurotoxic effects of MDMA. The authors concluded that in the absence of a fully functional dopaminergic system (such as at PND 21) and even in the presence of hyperthermia, which has been shown to be conducive to the expression of neurotoxicity, lasting reductions of 5-HT markers do not occur, hence supporting a role for DA in the neurotoxicity process.

The requirement of exogenous DA for neurotoxicity was confirmed in an in vitro study using a human serotonergic cell line, whereby programmed cell death induced by MDMA could be potentiated by elevation of extracellular levels of DA, but not 5-HT (Simantov and Tauber, 1997). Collectively, these and other similar reports establish the likelihood that DA is an important intermediate in MDMA-induced neurotoxicity.

MDMA elicits potent release of 5-HT and to a lesser extent DA. In vivo, however, the magnitude of DA release appears to greatly exceed what occurs in vitro. MDMA induced DA release has been shown to involve both carrier and impulse mediated release (Nash and Brodkin, 1991; Gudelsky and Nash, 1996; Yamamoto et al., 1995). Numerous reports have supported the involvement of a 5-HT_{2A} receptor-mediated process in the MDMA-induced release of DA and subsequent neurotoxicity (Schmidt et al., 1990a, 1992a&b). The 5-HT_{2A}-receptor mediated potentiation of dopaminergic transmission has been postulated to occur only during states of high serotonergic and dopaminergic transmission (Schmidt et al., 1992). For example, using microdialysis studies in the striatum, MDL-100,907, a 5-HT_{2A} receptor antagonist, was found to reduce MDMA-induced release of DA, although it failed to produce any significant effects on extracellular levels of DA by itself (Schmidt et al., 1994). Conversely, stimulation of 5-HT_{2A} receptors with the agonist R-DOI potentiated the

MDMA-induced DA release and consequent neurotoxic effects of MDMA (Gudelsky et al., 1994). Thus, in a situation known as "candy flipping" when a person ingests MDMA and also a drug like LSD (5-HT_{2A} agonist) they may be more likely to incur neurochemical damage. Additionally, studies from our group (Huang and Nichols 1993) support the hypothesis that amplification of DA synthesis via 5-HT_2 receptor activation might be responsible, at least in part, for the excessive DA release elicited by MDMA. These results support the idea that MDMA-induced serotonergic losses are dependent on the production of excessive extracellular dopamine that may be involved in the neurodegenerative mechanism.

Although the mechanism of DA involvement in 5-HT terminal degeneration is not yet understood, it has been speculated that DA might be taken up into the 5-HT terminal by the SERT, where the neurotransmitter is then deaminated by MAO-B to generate hydrogen peroxide, leading to impairments in the antioxidant machinery, membrane lipid peroxidation, and ultimate loss of 5-HT terminal integrity (Sprague and Nichols 1995). In further support of the involvement of ROS in the MDMA induced loss of 5-HT terminals, Jayanthi et al. (1999), using transgenic mice carrying an extra copy of h-SOD (superoxide dismutase) showed that MDMA (4 x 20 mg/kg) induced loss of 5-HT markers, decreases in antioxidant enzymes, and increases in membrane lipid peroxidation in the hippocampus and frontal cortex were dramatically reduced. In contrast, administration of MDMA to non-transgenic mice caused marked depletion of DA, DOPAC, and 5-HT, and led to membrane lipid peroxidation in the striatum and frontal cortex. The authors concluded that free radicals generated during MDMA

exposure might create a disturbance of the antioxidant system, resulting in the overproduction of free radicals with associated terminal damage and loss.

Consistent with the hypothesis that the DAT might act as an intermediary in the neurotoxicity process, Shankaran et al. (1999) recently showed that the DAT mediated release of DA might be an important source of hydroxy radical generation. Using microdialysis, they found that MDMA induced an increase not only in the extracellular concentration of DA, but also 2,3-dihydroxybenzoic acid (2,3-DHBA), a stable hydroxy radical adduct of salicylic acid. Both effects were attenuated by pretreatment with mazindol, a DAT inhibitor. Consistent with previous reports, mazindol pretreatment also attenuated long lasting reductions in 5-HT markers in the striatum. Hence, DA-mediated formation of hydroxy radicals might mediate, at least, in part, ROS generation and subsequent terminal loss produced by MDMA.

Thus, auto-oxidation and enzyme mediated oxidation of DA might be key processes in the loss of 5-HT axon terminals induced by MDMA. Therefore, pharmacological agents that attenuate MDMA-induced excessive dopaminergic transmission might be important in combating the persistent neurochemical damage elicited by MDMA.

The Role for GABA in Neuorotoxicity

As discussed above it is well established that attenuation of dopamine synthesis and release blocks MDMA-induced neurotoxicity in the striatum and frontal cortex (Gudelsky and Nash 1996). Baldwin et al., (1993) demonstrated that methamphetamine-induced DA release and subsequent neurotoxic effects on monoamine neurons in the rat striatum was attenuated by pretreatment with a sedative hypnotic and GABA-A agonist such as chlormethiazole. Thus, the authors concluded that GABA-mediated inhibition of striatal dopamine function could attenuate methamphetamine-induced loss of DA and 5-HT markers. In an analogous fashion, since methamphetamine and MDMA share the common characteristic of causing pronounced DA release, the neuroprotective effects of chlormethiozole (Colado et al., 1993), and pentobarbitone (Coaldo and Green, 1994) were tested in MDMA treated rats. The results showed that pretreatment with GABAergic agents attenuated MDMA-induced neurotoxicity. The neuroprotection afforded by these agents was hypothesized to involve inhibition of MDMA-induced DA release.

MDMA-induced perturbation of GABA function via indirect modulation of serotonin 5-HT_{2A} receptors by endogenously released 5-HT is also believed to mediate the neurotoxic effects of MDMA. For example, Yamamoto et al. (1995) showed that elevated extracellular DA was accompanied by a parallel reduction in GABA levels, particularly in regions that receive dual DA and GABA innervation, namely the striatum. The elevated DA was, however, reversed by pretreatment with ritanserin, a 5-$HT_{2A/2C}$ antagonist. The authors concluded that the elevated DA efflux induced by MDMA is

associated with disinhibition of the nigrostriatal dopaminergic output through a 5-HT_2 receptor-mediated suppression of striatonigral GABAergic negative feedback.

The ability of GABA mimetics to attenuate the neurotoxic effects of MDMA was believed to be due to their antagonistic effects on DA release. Recently, however, Colado et al. (1999d) re-evaluated the mechanism of the neuroprotective effects of chlormethiazole on acute MDMA-induced DA release and subsequent long-term reductions of 5-HT parameters, because some drugs attenuate damage due to their ability to induce hypothermia (Farfel and Seiden 1995; Malberg et al., 1996) or attenuate MDMA-induced hyperthermia (Colado et al., 1998). In light of the speculation that α-methyl-para-tyrosine could be neuroprotective because of the drug's inherent hypothermic effect, the authors re-examined their earlier data on haloperidol and chlormethiazole to clarify further the importance of DA in the neurodegenerative effects of MDMA.

The following studies raise some questions regarding the role of DA in the expression of MDMA-induced neurotoxicity. For example, Malberg et al. (1996) demonstrated that MDMA-induced decreases in 5-HT and 5-HIAA levels in the frontal cortex, somatosensory cortex, striatum, and hippocampus were attenuated by pretreatment with α-methyl-para-tyrosine (AMPT). Upon warming the animals to prevent hypothermia, however, the neuroprotective effect of AMPT was lost. The authors hypothesized that a decrease in core temperature, independent of any inhibitory effect on MDMA-induced DA synthesis or release, might be the underlying mechanism of AMPT neuroprotection. If reduced GABAergic input into the substantia nigra (Yamamoto et al., 1995) was the factor underlying MDMA-induced DA release, then

reinstatement of GABAergic transmission should have attenuated the facilitatory effects of MDMA on DA release. As evidence against this hypothesis, however, chlormethiazole had no effect on the acute MDMA-induced increased DA in striatal dialysate of rats. Additionally, a concomitant induction of hypothermia was observed in chlormethiazole-pretreated rats. Upon elevation of body temperature, dramatic reduction in the magnitude of neuroprotection provided by chlormethiazole was observed. This study raised the possibility that hypothermia may prevent MDMA-induced neurotoxicity, and that modulation of MDMA-induced changes in dopaminergic transmission may not be involved in the neuroprotective mechanism of chlormethiazole.

In another set of studies examining the neuroprotective efficacy of pentobarbitone, a compound which is believed to possess a similar pharmacological profile to that of chlormethiazole, Colado et al. (1999a) demonstrated that pentobarbitone attenuated MDMA-induced loss of 5-HT markers following concurrent administration of MDMA and pentobarbitone. When the body temperature of the rats receiving the drug combination was kept elevated, however, the neuroprotective effect of pentobarbitone was lost. The authors concluded that the hypothermic effect, rather than an intrinsic neuroprotective effect, might underlie the neuroprotective action of $GABA_A$ agonists.

Despite studies demonstrating that chlormethiazole's neuroprotective effects might be derived from its hypothermic effect, the question that remained unanswered was whether the hypothermic response elicited by chlormethiazole might have also ameliorated the free radical generation induced by MDMA. Previous studies have shown that hyperthermia potentiates free radical formation in the brain. Conversely, hypothermia reduces the magnitude of the formation of free radicals (Globus et al., 1995,

Kil et al., 1996). Moreover, there is substantial evidence to support the idea of formation of free radicals (via metabolic oxidation of DA, as well as formation of catechol and quinone metabolites of MDMA) in MDMA-induced neurotoxicity (Sprague et al., 1995; Colado and Green, 1995). In fact, Colado et al. (1999c) investigated the neuroprotective effects of chlormethiazole on MDMA-induced free radical formation using microdialysis studies and measured formation of two ROS adducts 2,3-DHBA and 2,5-DHBA using microdialysis studies. Chlormethiazole produced significant reduction in MDMA-induced hyperthermia and also marked reductions in the magnitude of ROS generation. The inhibitory effect of chlormethiazole on MDMA-induced generation of ROS was lost, however, upon elevation of body temperature, to that of rats treated with MDMA alone. The authors concluded that chlormethiazole's neuroprotective effects are not due to any free radical scavenging property, but to the drug's ability to induce hypothermia. Regardless of the mechanism of action of GABAmimetics, it is becoming increasingly clear that hypothermia might exert a negative modulatory effect on the neurotoxic effects of MDMA.

The studies summarized above demonstrate the feasibility of using GABAergic agents to block the neurodegenerative effects of MDMA. The current results highlight the importance of body temperature in modulating the neurotoxic effects of MDMA, independent of the drug's ability to attenuate MDMA-induced increase in DA release and ROS generation. These studies raise the possibility that the rise in body temperature following physical exertion at "rave" parties might facilitate the expression of the neurotoxic effects of MDMA. Hence, in emergency situations where patients with drug-induced intoxication together with hyperthermia are admitted for treatment,

GABAergic agents might serve as an effective agent not only in attenuating the possibility of MDMA-induced neurotoxicity, but also the drug induced hyperthermia.

Summary

In summary, MDMA is a 5-HT neurotoxin that mediates the selective loss of 5-HT terminals, while leaving the dopaminergic and the noradrenergic neurons intact. Compelling preclinical data suggest that psychological problems encountered by repeated MDMA users might be attributed to the loss of 5-HT function. In this context, recent PET studies support the preclinical studies and show that human users may have marked reductions in the SERT even after prolonged drug abstinence. There is substantial evidence to show the interaction of multiple neurotransmitter systems including 5-HT, DA, and GABA in MDMA-induced neurotoxicity. Many studies suggest that attenuation of MDMA-induced increased dopaminergic transmission might prevent long-term reductions of 5-HT markers. In addition, hypothermia might also be neuroprotective. Thus, agents that block MDMA-induced dopaminergic transmission, along with inhibition of hyperthermia might be effective in preventing the neurotoxic effects of MDMA. Taken together, several neurochemical events (figure 2) converge on 5-HT neurons following MDMA exposure to render them vulnerable to degenerative changes.

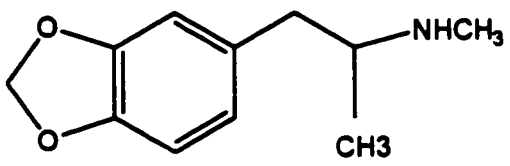

Figure 1. Structure of MDMA

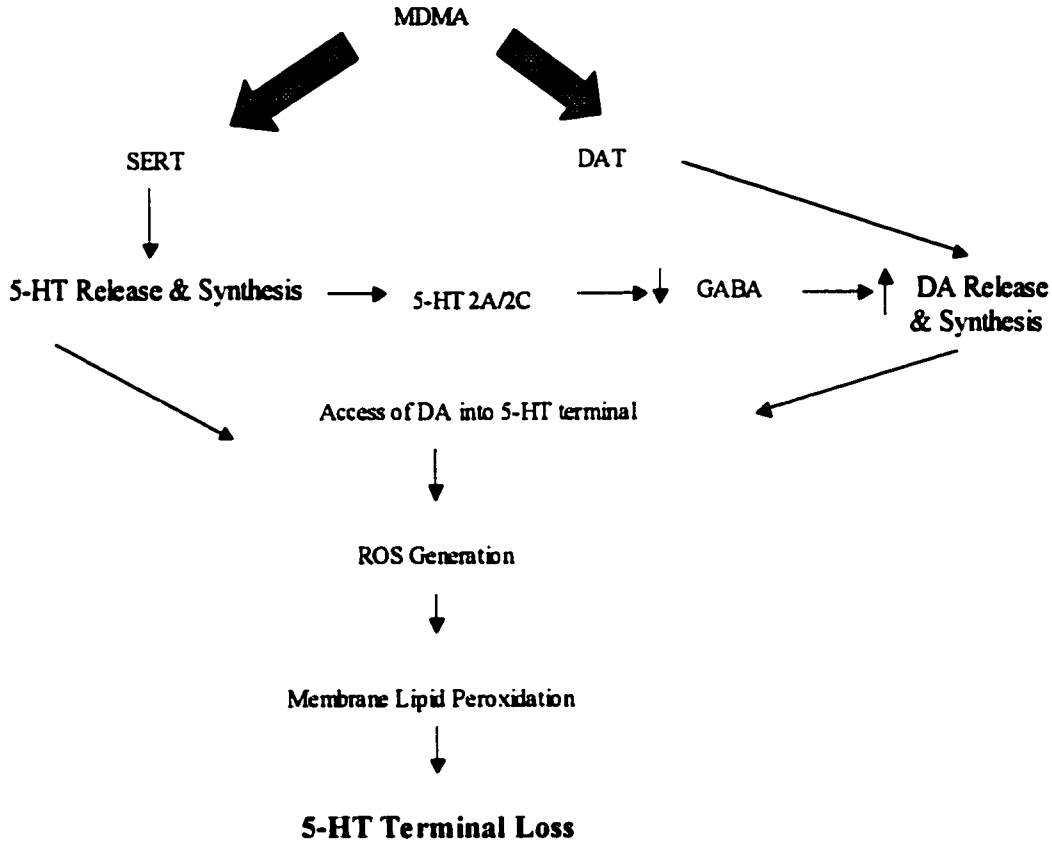

Figure 2. Putative Model For MDMA-Induced Serotonin Terminal Neurodegeneration

MATERIALS METHODS AND RESULTS

The role of GABA in MDMA-induced 5-HT terminal degeneration

Hypothesis:

MDMA-induced reduction in GABAergic neurotransmission might mediate, at least in part, the long-term reductions in 5-HT by indirectly modulating dopaminergic activity. Additionally, attenuation of MDMA-induced hyperthermia might protect against neurotoxicity.

Experiment one:

Rationale:

Serotonin mediated disinhibition of the striatonigral GABAergic pathway produced by the activation of 5-$HT_{2A/2C}$ receptors has been implicated in the enhanced activity of the nigrostriatal dopaminergic pathway following MDMA administration (Yamamoto et al., 1995). To understand the importance of GABA in MDMA induced serotonergic neurotoxicity we utilized gamma vinyl GABA (GVG) an irreversible suicide inhibitor of GABA-transaminase (GABA-T). Additionally, several studies have reported that hypothermia is an important functional response associated with several GABAergic

agents (Colado et al., 1998, Colado et al., 1999a). Therefore, we determined the effects of GVG pretreatment on MDMA-induced hyperthermia.

Experimental Design:

Thirty animals were randomly distributed to five groups (n = 5-7). Treatments were given twice daily, for four days. The experimental groups were as follows:

Group I	Saline (control)
Group II	GVG (500 mg/kg, i.p.)
Group III	MDMA (20 mg/kg, s.c. b.i.d. for 4 days)
Group IV	GVG-Acute + MDMA (GVG given 3 h prior to MDMA) (GVG-A/MDMA
Group V	GVG-Long term + MDMA (GVG given 24 h prior to MDMA) (GVG-L/MDMA

All doses of MDMA and saline were administered subcutaneously, whereas GVG was given intraperitoneally. GVG was administered either 3 hours (MDMA + GVG-A) prior to MDMA or given as a total of 2 doses (MDMA + GVG-L): with the first dose of GVG given 24 h prior to the first dose of MDMA and the second dose given 48 hours following the first dose of GVG (or 24 h after MDMA).

Subjects:

Studies described in this thesis were carried out in accordance with protocols approved by the Purdue University Animal Care and Use Committee.

Male-Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 200-230 grams were used in this experiment. Animals were individually housed. Room temperatures were maintained at 22 to 24 °C and a 12/12 light/dark cycle was utilized. Food and water were provided *ad libitum.*

Materials:

MDMA hydrochloride was synthesized in our laboratory following the methods of Nichols et al., (1986). GVG was generously provided by Dr. Stephen Dewey, (Brookhaven National Laboratory, Long Island, NY). Drug solutions were dissolved in saline such that the volume of injection was 1 ml/kg.

Methods:

Rectal temperature was recorded using a digital thermometer (CMA/150 Carnegie Medicin; Stockholm, Sweden). The probe was lubricated with K-Y jelly and inserted 3 cm into the rectum and the rat was gently held. A steady readout was obtained following 30 s of probe insertion. Body temp was recorded every hour for the next 7 h.

Results:

Figure 3 shows the effect of GVG on rectal temperature in MDMA treated rats. MDMA (20 mg/kg, s.c.) produced a sustained hyperthermic response of approximately 1.2-1.8 °C that lasted for at least 7 h. In rats that received GVG 3 h or 24 h prior to MDMA, an attenuation of MDMA-induced hyperthermia was evident. In rats that

received GVG, acutely, however, a gradual reduction in the hypothermic response was observed at the end of 7 h.

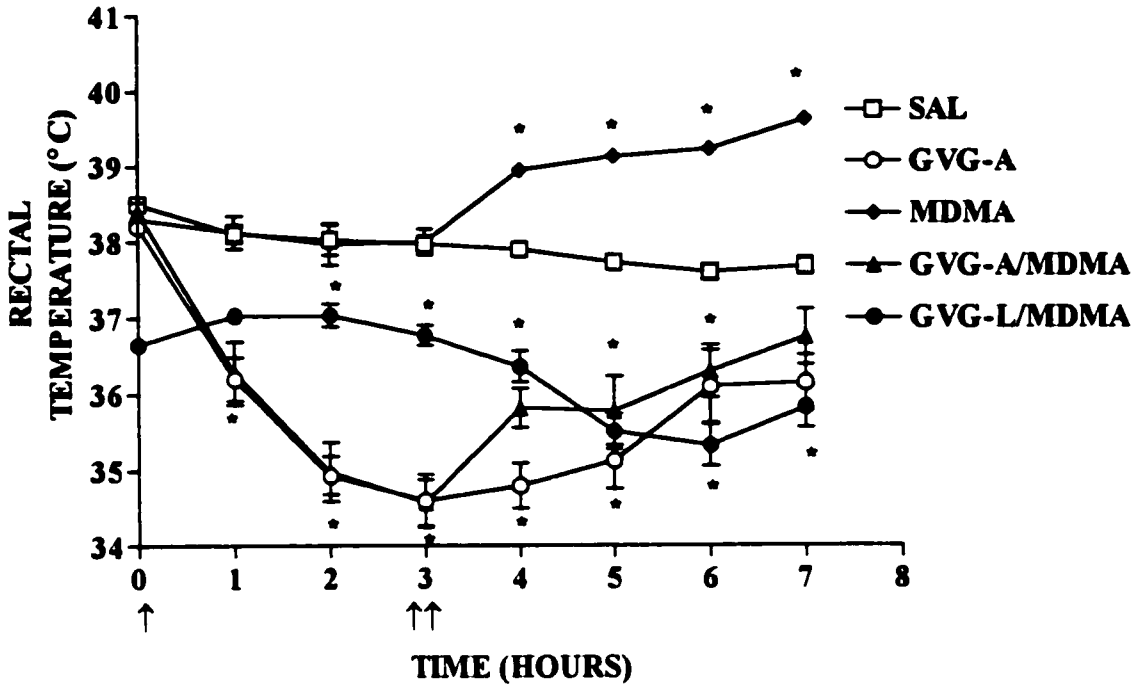

Figure 3. Effects of GVG and MDMA on core temperature in rats. Rats were administered either GVG (500 mg/kg) or saline (1 ml/kg) 3 h (GVG-A; GVG-A/MDMA) or 24 h prior to MDMA (GVG-L/MDMA). The single arrow represents GVG injection (GVG-A); Double arrows represent MDMA injections. GVG alone produced pronounced hypothermia, whereas GVG plus MDMA caused a prolonged and significant (* $p < 0.05$, ANOVA, Newman Keuls post test) decrease in temperature in comparison with MDMA and saline (SAL). Each value is the mean ± SEM for 5-8 animals.

Experiment two:

Rationale:

Having demonstrated that GVG attenuates MDMA-induced hyperthermia, we decided to examine whether the drug-induced hypothermia modulated long term reductions in 5-HT markers induced by MDMA.

Experimental Design:

Thirty animals were randomly distributed to five groups (n = 5-7). Treatments were given twice daily, for four days. The experimental groups were as follows:

Group I	Saline (control)
Group II	GVG (500 mg/kg, i.p.)
Group III	MDMA (20 mg/kg, s.c. b.i.d. for 4 days)
Group IV	GVG-Acute + MDMA (GVG given 3 h prior to MDMA) (GVG-A/MDMA
Group V	GVG-Long term + MDMA (GVG given 24 h prior to MDMA) (GVG-L/MDMA

All doses of MDMA were administered subcutaneously; all other drugs were given intraperitoneally. GVG was administered either 3 hours prior to saline or MDMA (Group-IV); or given as a total of 2 doses, with the first dose of GVG given 24 h prior to the first dose of MDMA and the second dose given 48 hours following the first dose of GVG (Group-V). All the animals were sacrificed 7 days after the last dose of MDMA.

Upon sacrifice, rat brains were dissected on a chilled petri dish according to the procedure of Glowinski and Iversen (1966). The two hemispheres were individually dissected into separate frontal cortex, hippocampus, and striatum and quickly frozen in liquid nitrogen and stored at –70 °C for future biochemical measurements.

Subjects:

Animals were housed under conditions similar to those described for experiment one.

Materials:

MDMA hydrochloride was synthesized in our laboratory following the methods of Nichols et al., (1986). GVG was generously provided by Dr. Stephen Dewey, (Brookhaven National laboratory, Long Island, NY). Drug solutions were dissolved in saline such that the volume of injection was 1 ml/kg. [^{3}H]-paroxetine was purchased from New England Nuclear (Boston, MA) at a specific activity of 23 Ci/mmol. Fluoxetine hydrochloride was purchased from Sigma Chemical Company (St Louis, US). 5-HT and 5-HIAA were purchased from Sigma chemical company (St Louis, MO). Ecolite scintillation fluid was obtained from ICN (Costa Mesa, CA). All other materials were purchased from Purdue University pharmacy stores.

Methods:

[^{3}H]-Paroxetine Binding procedure:

A modified procedure of Marcusson et al. (1988) was employed to measure the density of 5-HT uptake sites. A buffer consisting of 50 mM Tris HCl with 120 mM NaCl and 5 mM KCl at pH 7.4 was employed for both tissue homogenization and for incubations. Tissue samples were weighed and homogenized in 5 ml of the buffer described above with a Brinkman polytron (setting 6, 2 x 20 s). The homogenate was centrifuged twice at 30,000 x g for 10 min with an intermittent wash and the pellet was resuspended in the same volume of the buffer described above. Since it has been previously reported that only the B_{max} and not the K_D values are altered after MDMA treatment (Battaglia et al., 1987), it is possible to estimate the number of 5-HT uptake sites with a saturating concentration of [^{3}H]paroxetine (K_D=0.01 nM) (Battaglia et al., 1988). Therefore for this experiment we used only a high single concentration (1nM) of [^{3}H]-paroxetine. Specific binding was defined as that displaceable with 1 μM fluoxetine. Incubations were started by adding 150 μl of tissue homogenate to each tube to give a final volume of 1.65 ml. Tubes were allowed to equilibrate at 24 °C for 1h before adding 4 ml of ice cold buffer and filtering through Whatman GF/C filters using a Brandel cell harvester. The tubes were washed twice with 5 ml of ice cold buffer and the filters were placed in plastic vials containing 10 ml scintillation cocktail. The vials were allowed to stand overnight prior to counting at an efficiency of 40%.

HPLC analyses of the indole levels in forebrain regions:

Subjects were sacrificed by decapitation and the forebrain regions (striatum, hippocampus, and frontal cortex) were assayed for content of 5-HT and 5-HIAA by HPLC-EC. Briefly, following rapid dissection on ice, the tissue was suspended in 0.5 ml of buffer containing 0.1M $HClO_4$, 0.05% Na_2EDTA and 0.1% $Na_2S_2O_5$ and homogenized using a motor-driven teflon pestle. Subsequently, the samples were centrifuged at 14,000 x g for 5 min in a tabletop centrifuge. The supernatant was then added to a 22 micron nylon filter (Rainin, Woburn, MA) and centrifuged at the same speed for 10 min. The filtrate was analyzed for monoamines and their metabolites by separation on an ESA MD-150 column with a flow rate of 0.7 ml/min using ESA MD-TM mobile phase. The HPLC-EC system consisted of an ESA pump (model 582) linked to an autosampler (Tosohaas, Philadelphia, PA) and an electrochemical detector consisting of a Coulochem 5200A electrochemical detector with a Coulochem 5011 detector cell. The Coulochem was operated in screen mode with the potential of the screening electrode set at 750 mV and the potential of the quantitating electrode set at - 200 mV. The applied potential at the working electrode of the electrochemical detector was set at 610 mV. The current produced was integrated using Rainin software for determination of tissue 5-HT and 5-HIAA levels.

All measurements were based on wet tissue weight. The average of saline treated rats (n = 5-7) was considered as the control and defined as 100%. All values presented are expressed as percentages of controls. Differences between treatment groups were

evaluated by an ANOVA with Student-Newman-Keuls post - hoc test. Significance was set at $p < 0.05$.

Results

Figure 4 depicts the effect of GVG pretreatment on the long-term neurodegenerative effects of MDMA. MDMA (20 mg/kg, b.i.d, 4 days) produced a significant reduction in the density of [^{3}H]-paroxetine labeled sites in the cortex, hippocampus, and striatum ($p < 0.01$). However, GVG pretreatment attenuated the MDMA-induced loss of 5-HT uptake sites in all the forebrain regions.

MDMA produced dramatic reductions (50-65%) in 5-HT and 5-HIAA levels in the cortex, hippocampus, and striatum one week following the last dose of MDMA. GVG alone had no effect on 5-HT and 5-HIAA in the hippocampus and striatum. In contrast, a significant ($p < 0.01$) decrease of monoamine levels was observed in the cortex. GVG, in combination with MDMA provided substantial protection against MDMA-induced reduction in 5-HT and 5-HIAA in the forebrain regions.

Figure 4. Effects of MDMA (20 mg/kg, s.c, twice daily, for 4 days) and GVG (500 mg/kg) on the serotonergic markers seven days following the last dose of MDMA. Saline or 500 mg/kg GVG was injected intraperitoneally either 3 h prior to MDMA (GVG; GVG-A/MDMA) or given 24 h before the first dose of MDMA, followed by the second dose of GVG given 48 h later (GVG-L/MDMA). Saline or MDMA was administered subcutaneously twice daily every 12 h for 4 days. Animals were sacrificed one week after the last dose. Values are presented as the mean ± SEM for N = 5-7. Saline control values for frontal cortex (A), hippocampus (B), and striatum (C) are: frontal cortex: 5-HT 204.14 ± 15.27, 5-HIAA 184.08 ± 12.57 pg/mg wet wt.; and SERT 14.0 ± 0.29 fmol/g wet wt; hippocampus: 5-HT 215.43 ± 10.45, 5-HIAA 227.53 ± 11.93 pg/mg wet wt.; and SERT 11.90 ± 0.64 fmol/g wet wt; and striatum 5-HT 220.48 ± 12.11, 5-HIAA 329.19 ± 21.85 pg/mg wet wt.; and SERT 14.27 ± 0.70 fmole/mg wet wt. Indicates $^{a}p < 0.001$, compared to saline, $^{b}p < 0.01$, compared to saline, $^{c}p < 0.05$, compared to MDMA, $^{d}p < 0.01$,compared to MDMA, $^{e}p < 0.05$ (ANOVA, Newman Keuls post test) compared to saline.

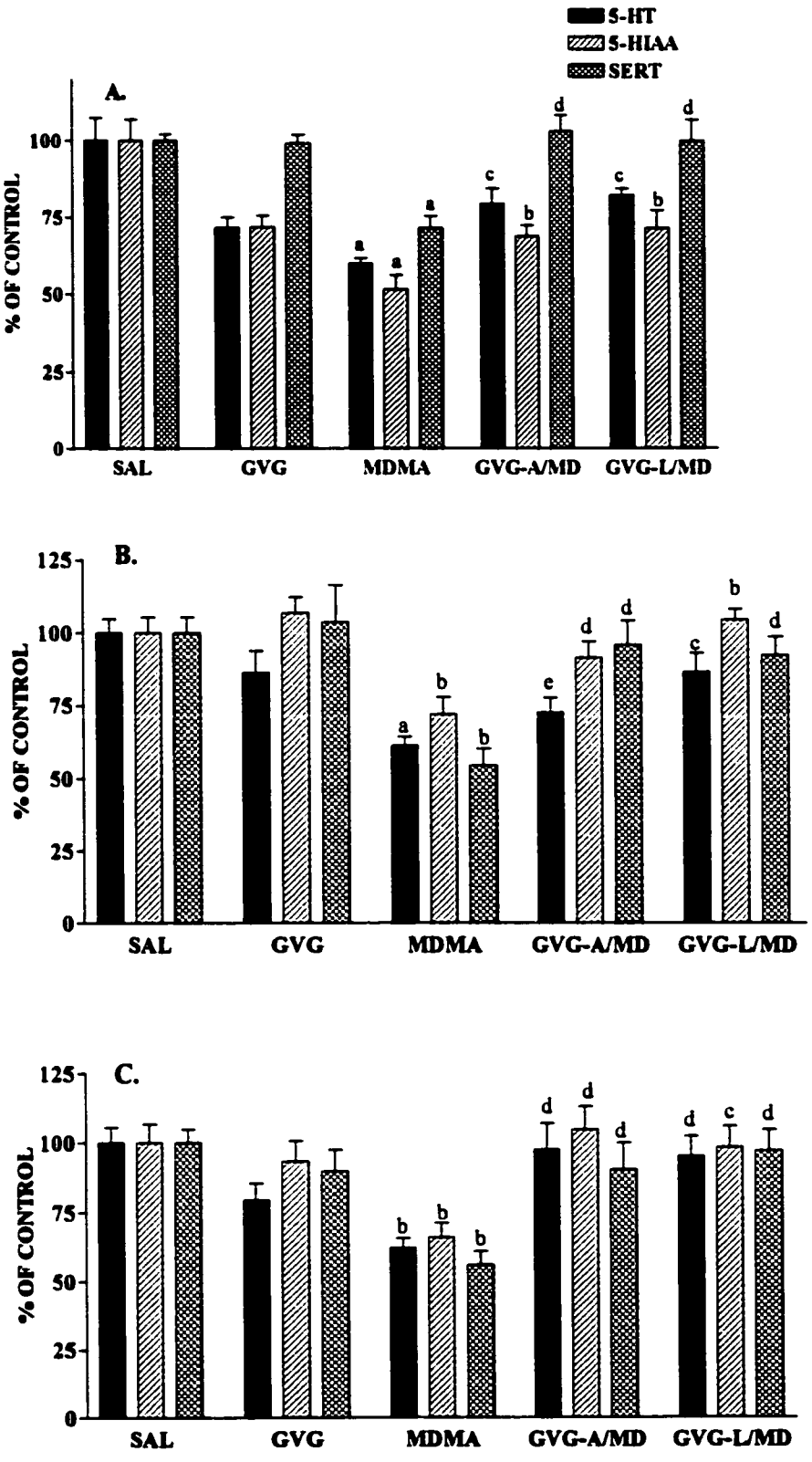
5-HT
5-HIAA
SERT
A.
% OF CONTROL
100
75
50
25
0
SAL
GVG
MDMA
GVG-A/MD
GVG-L/MD
B.
125
C.

Figure 4

GABA-B receptor modulation in MDMA neurotoxicity

Hypothesis:

If suppression of GABAergic neurotransmission is an important attribute of the MDMA-induced neurotoxicity process, then prior activation of the tonic regulatory negative feedback system via GABA-B receptor activation should offset MDMA's inhibitory effect on extracellular GABA levels and subsequent neuronal degeneration. Attenuation of MDMA-induced hyperthermia might also contribute to neuroprotection.

Experiment three:

Rationale:

To elucidate further the neurochemical mechanism underlying GVG's neuroprotective effects, the role of the GABA-B receptor was probed in particular because it had been shown to be involved in the tonic regulation of the nigrostriatal dopaminergic system (Santiago et al., 1993). Also, since hypothermia has been shown to be an inherent pharmacological effect of several GABAmimetics (Colado et al., 1994, Colado et al., 1998, Colado et al., 1999) we examined the effect of baclofen, a prototypical GABA-B agonist on MDMA-induced hyperthermia.

Experimental Design:

Thirty-two animals were randomly assigned to four different treatment groups. The groups were as follows:

Group I Saline

Group II Baclofen (18 mg/kg, i.p.)

Group III MDMA (40 mg/kg, s.c.)

Group IV Baclofen/ MDMA (baclofen given 45 minutes prior to MDMA)

Baclofen pretreatment was administered 45 minutes prior to MDMA. The rectal temperature of rats was monitored for the next four hours following MDMA.

Subjects:

Animals were housed, maintained and handled as in experiment one.

Materials:

Baclofen hydrochloride was purchased from RBI (St Louis, MO, USA). All compounds were dissolved in 0.9 % saline or in double distilled water. Control animals were administered saline. All drugs were administered in a volume of 1 ml/kg.

Methods:

Rectal temperature was monitored in the manner described in experiment one.

Results:

Administration of MDMA (single injection, 40 mg/kg, s.c.) produced sustained hyperthermia that prevailed throughout the experimental period (Figure 5). Conversely,

when baclofen (18 mg/kg, i.p.) was injected 30 minutes before MDMA, this rise in body temperature was completely abolished. Indeed, a hypothermic response was evident in rats receiving the combination of MDMA and baclofen. Additionally, baclofen alone produced a 2 °C drop in rectal temperature.

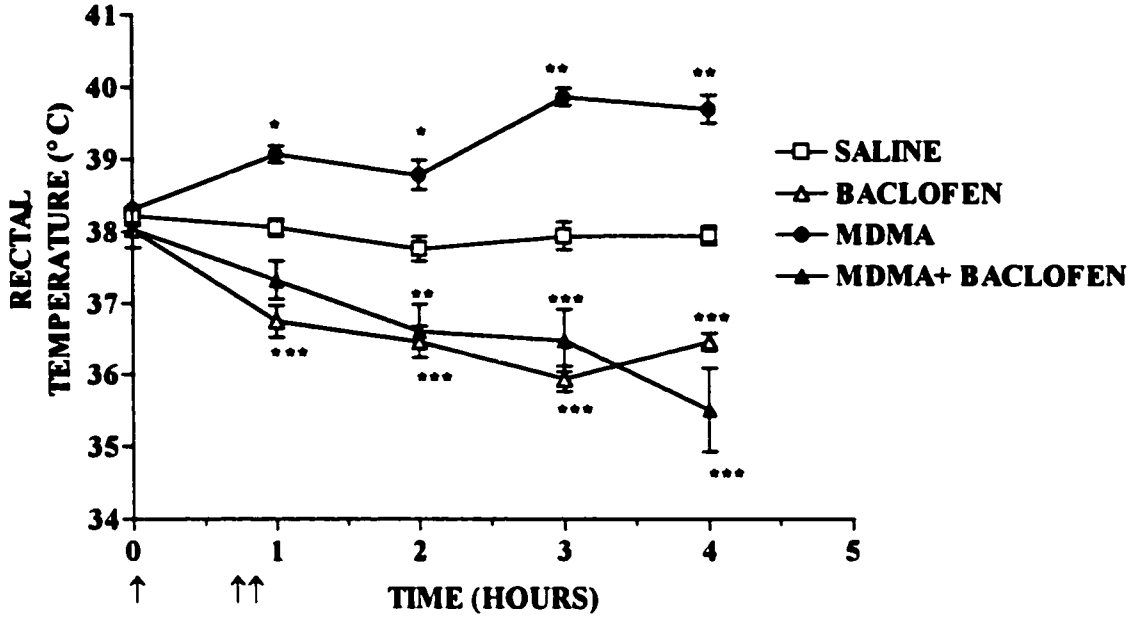

Figure 5. Effect of baclofen on MDMA-induced alteration in body temperature of rats. Rectal temperature of rats administered baclofen (18 mg/kg, i.p.) or saline at time zero, with MDMA (40 mg/kg, s.c.) or saline injected 45 minutes later. The single arrow represents baclofen injection; double arrows represent MDMA injections. MDMA produced a significant increase in body temperature (*$p < 0.05$, **$p < 0.01$, ANOVA

Newman Keuls post test), compared with the saline treated group. However, administration of baclofen prior to MDMA attenuated the hyperthermic response elicited by MDMA (***$p < 0.001$, ANOVA, Newman Keuls post test). Baclofen alone produced pronounced hypothermia in comparison to saline treated rats.

Experiment four:

Rationale:

Previously it was demonstrated that MDMA potentiates DA synthesis (Huang et al., 1994) via a 5-HT_2 receptor-mediated mechanism (Gudelsky et al., 1995), resulting in accentuated acute release of DA. Evidence has accumulated that GABA action on GABA-B receptors might be involved in the regulation of tyrosine hydroxylase activity in nigrostriatal dopaminergic terminals (Arias-Montano et al., 1991). We sought to investigate whether baclofen blocks the MDMA-induced acute increase in DA in the striatum and cortex.

Experimental Design:

Thirty-two animals were randomly divided into four treatment groups as described in experiment three. Rats were sacrificed 3 h following MDMA exposure and the striatum and frontal cortex were assayed for changes in DA and its metabolite DOPAC.

Subjects:

Rats were housed, maintained, and handled as described in experiment one.

Materials:

DA and DOPAC were purchased from Sigma chemicals (St Louis, MO). All other materials were obtained from the same sources as listed in experiment two.

Results:

Effects of baclofen pretreatment on MDMA-induced acute alteration of DA and DOPAC levels in the striatum and cortex 3h after MDMA administration are shown in Table 1. Baclofen (18 mg/kg, i.p.) was injected 45 minutes prior to MDMA (40 mg/kg, s.c.). Three hours after MDMA treatment with MDMA alone, striatal dopamine content was increased by approximately 32%. In contrast, animals that received baclofen prior to MDMA showed significant attenuation of the MDMA-induced increase in DA content in striatum ($p < 0.05$), but not in the cortex. In fact, cortex showed a significant ($p < 0.01$) increase in tissue DA content. There were no significant changes in tissue DA concentration, however, in rats that received baclofen alone.

MDMA produced a significant (~60%, $p < 0.001$) reduction in DOPAC content in both the striatum and the cortex. In rats that received both baclofen and MDMA however, there were no significant changes in tissue DOPAC content, when compared to the MDMA treatment group.

Table 1. The acute effects of MDMA alone and with baclofen pretreatment on cortical and striatal DA and DOPAC content 3h after drug administration. Rats were administered baclofen 45 minutes before saline or MDMA administration, DA and DOPAC content were determined 3h later. The concentration of DA and DOPAC in the striatum and cortex is expressed as ng g^{-1} tissue
Significantly different from saline: $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$; different from MDMA, significantly different from MDMA: $^{d}p < 0.01$, $^{e}p < 0.05$

TREATMENT	STRIATUM		CORTEX	
	DA (% of control)	DOPAC (% of control)	DA (% of control)	DOPAC (% of control)
SALINE	100 ± 4.63 (9773 ± 452.20)	100 ± 11.42 (1466.76 ± 167.51)	100 ± 6.03 (22.37 ± 1.35)	100 ± 7.58 (18.21 ± 1.38)
BACLOFEN	104.84 ± 3.68	99.87 ± 5.01	134.69 ± 14.07	135.2 ± 13.25
MDMA	132.03 ± 5.85^{c}	34.12 ± 3.85^{c}	196.02 ± 17.03^{b}	23.28 ± 2.94^{c}
BACLOFEN + MDMA	119.17 ± 3.63^{e}	43.96 ± 3.23^{c}	264.99 ± 25.31^{d}	12.55 ± 2.01^{c}

Experiment five:

Rationale:

Previously, it was demonstrated that baclofen exerts an inhibitory effect on potassium stimulated 5-HT release from cortical slices (Bowery et al., 1987). In this experiment, we examined the ability of baclofen to attenuate MDMA induced acute reductions in tissue 5-HT and 5-HIAA content.

Experimental Design:

The experimental design utilized in experiment four was duplicated for this study.

Subjects:

Animals were housed, and maintained as noted in experiment four.

Materials and Methods:

The biogenic amine analysis was carried out in a fashion identical to that used in experiment two.

Results:

Three hours following MDMA administration (figure 6), 5-HT and 5-HIAA levels were significantly reduced in the cortex and the striatum ($p < 0.001$). Baclofen alone had no significant effect on tissue monoamine content in either region. Both cortex and striatum from baclofen pretreated rats showed a trend towards protection against MDMA's neurodegenerative effects, however, the effect only reached statistical significance in the cortex.

Figure 6. Effects of MDMA (single injection, 40 mg/kg, s.c.) and baclofen (18 mg/kg, i.p.) on acute 5-HT and 5-HIAA tissue content 3 hours after treatment. The tissue content is reported as a percentage of control. Saline control values were: (A), cortex 5-HT 281.80 ± 19.24, 5-HIAA 417.46 ± 17.09 pg/mg wet wt; (B), striatum 5-HT 231.26 ± 13.21, 5-HIAA 124.52 ± 12.09 pg/mg wet wt. Each value is the mean of 5-7 animals. Significantly different from saline treated rats: $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$. Significantly different from MDMA treated rats, ^{d} $p < 0.01$ (ANOVA, Newman Keuls post test).

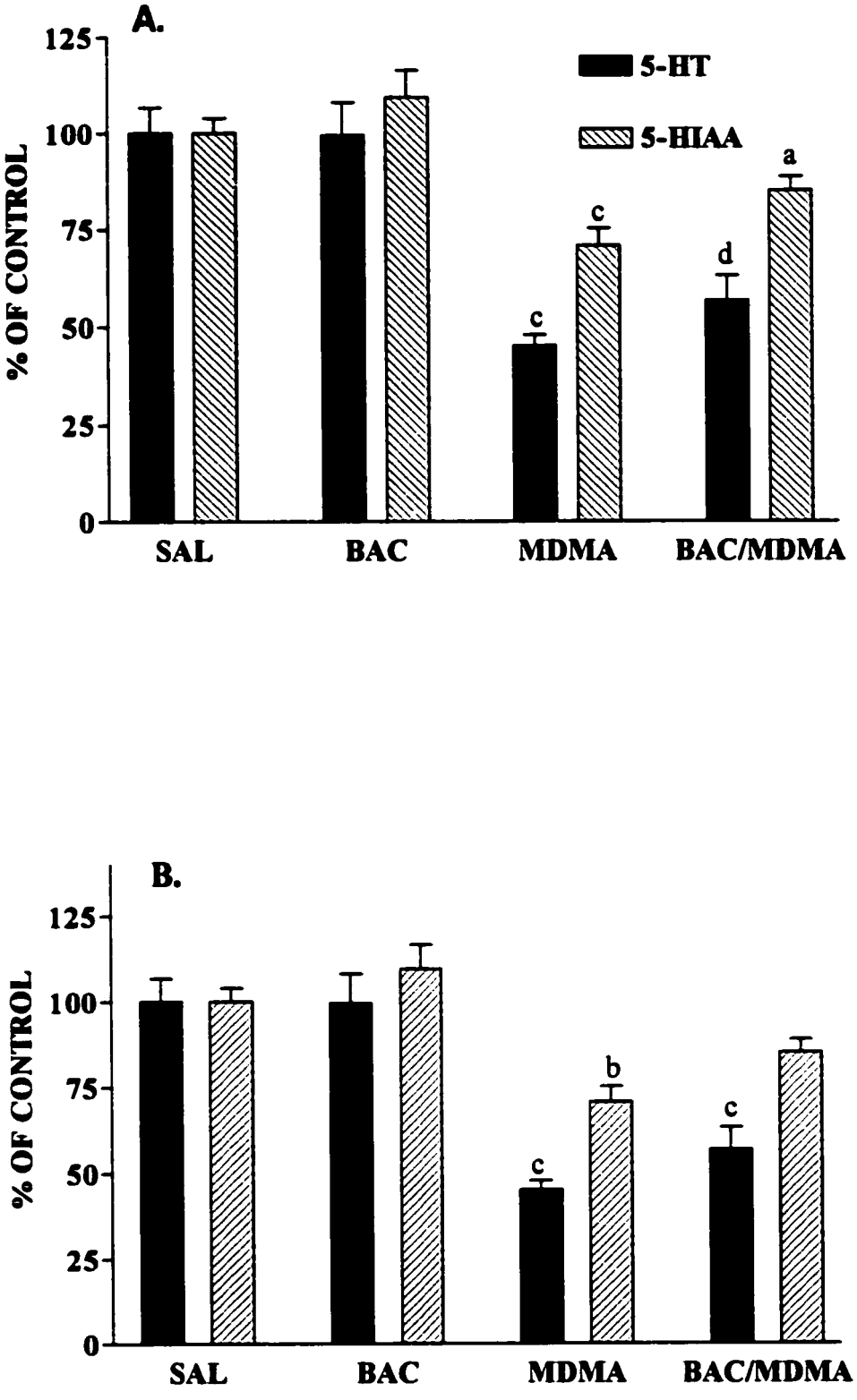
A.
125
100
75
50
25
0
% OF CONTROL
5-HT
5-HIAA
a
c
c
d
SAL
BAC
MDMA
BAC/MDMA
B.
125
100
75
50
25
0
% OF CONTROL
b
c
c
SAL
BAC
MDMA
BAC/MDMA

Figure 6

Experiment six:

Rationale:

Several studies have reported that agents that modulate MDMA-induced increases in dopaminergic transmission can prevent the long term neurodegenerative effects of MDMA (Schmidt et al 1987., Nash et al.,1991). Furthermore, abolition of the MDMA-induced hyperthermic response has been shown to antagonize the persistent loss of 5-HT parameters induced by MDMA. Thus, based on the observation that baclofen exerts a negative modulatory effect on: 1) MDMA-induced hyperthermia, 2) MDMA-induced increases in striatal DA content and 3) MDMA-induced reductions in acute cortical monoamine content, our objective was to assess the magnitude of MDMA-neurotoxicity in the forebrain regions of rats receiving a combination of baclofen and MDMA.

Experimental Design:

Thirty-two animals were randomly assigned to four different treatment groups. The groups were as follows:

Group I	Saline
Group II	Baclofen (18 mg/kg, i.p.)
Group III	MDMA (40 mg/kg, s.c.)
Group IV	Baclofen/ MDMA (baclofen given 45 minutes prior to MDMA)

Baclofen was administered 45 minutes prior to MDMA. All animals were sacrificed 2 weeks following treatment.

Subjects:

Animals were housed, maintained and handled as in experiment one.

Materials and Methods:

Drugs were obtained from sources noted above. All compounds were dissolved in 0.9 % saline or water. Control animals were administered saline. All drugs were administered in a volume of 1 ml/kg.

Methods:

All the methods utilized in this study were identical to the methods employed in experiment two.

Results:

A single dose of MDMA (40 mg/kg) led to a pronounced loss of 5-HT, 5-HIAA, and SERT density in the cortex, hippocampus, and striatum two weeks following MDMA injection (figure 7). In rats that were pretreated with baclofen, MDMA-induced loss of 5-HT markers was dramatically attenuated. Baclofen alone failed to exert any significant effect on serotonergic markers in any of the forebrain regions assayed.

Figure 7. Effect of baclofen (18 mg/kg, s.c.) administered 45 minutes before MDMA (40 mg/kg, i.p.) on serotonergic markers in the cortex (A), hippocampus (B), and striatum (C) two weeks later. Results shown as means ± S.E.M, n = 7-11. Saline control values were: cortex 5-HT 417.93 ± 15.61, 5-HIAA 271.87 ± 8.49 pg/mg wet wt; SERT 13.15 ± 0.54 fmole/g wet wt; hippocampus 5-HT 419.74 ± 24.81, 5-HIAA 334.39 ± 21.05 pg/mg wet wt SERT 10.51 ± 0.43 fmole/mg wet wt; striatum: 5-HT 298.33 ± 18.11, 5-HIAA 342.84 ± 19.36 pg/mg. Indicates significance: Different form saline control: $^{a}p < 0.001$. Different from MDMA: $^{b}p < 0.05$, $^{c}p < 0.01$, $^{d}p < 0.001$ (ANOVA, Newman Keuls post-test).

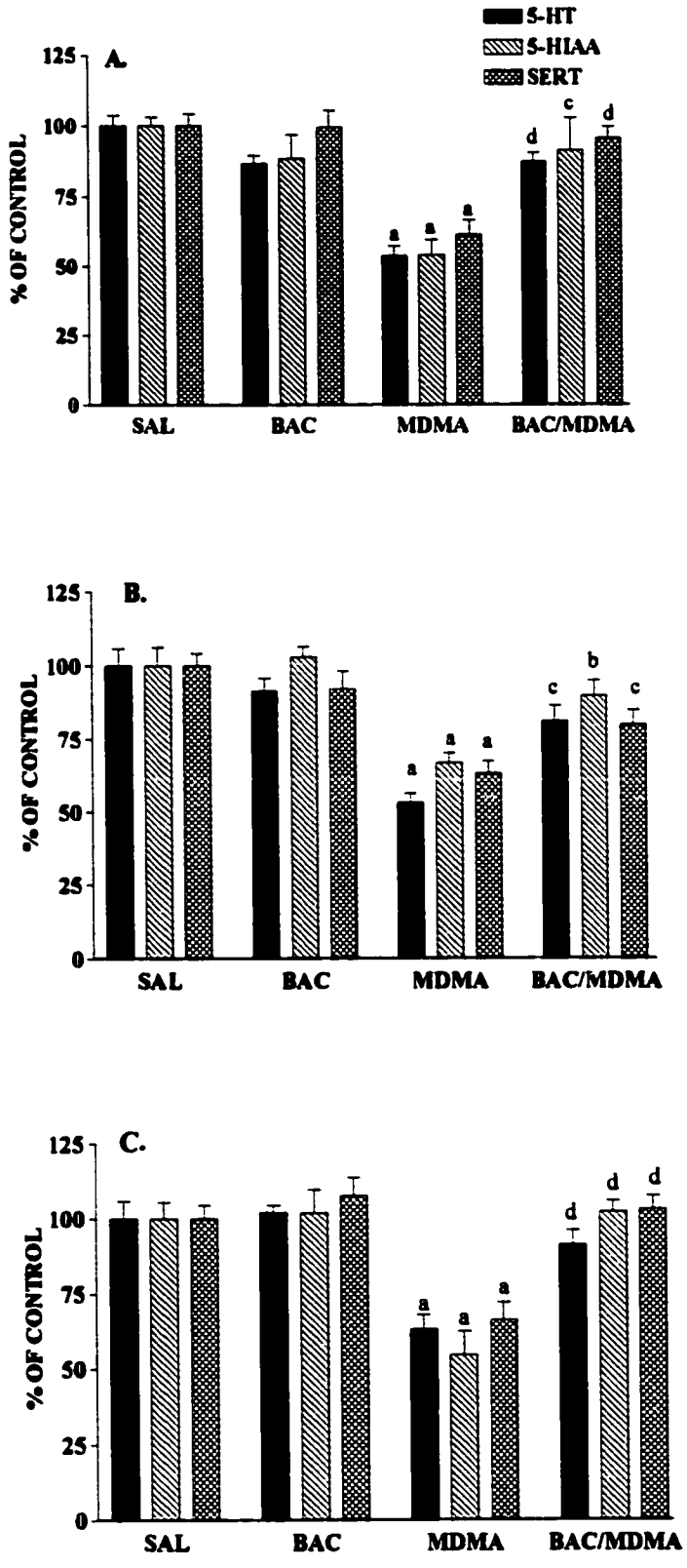
A.
B.
C.
5-HT
5-HIAA
SERT
% OF CONTROL
125
100
75
50
25
0
SAL
BAC
MDMA
BAC/MDMA
a
b
c
d

Figure 7

Effect of GABA-B Receptor Antagonism on MDMA-induced Neurotoxicity

Hypothesis:

Antagonism of the GABA-B receptor prior to MDMA should potentiate the drug-induced hyperthermia and subsequent neurodegenerative response provided reduced GABAergic neurotransmission is an important determinant of MDMA-induced neurotoxicity.

Experiment seven:

Rationale:

In the previous study, baclofen in combination with MDMA produced attenuation of MDMA-induced acute serotonergic deficits and hyperthermia. For this experiment, we utilized SCH-50911, a selective antagonist of GABA-B receptor (Ashby et al., 1999) to elucidate the effects of inhibition of GABA-B receptor on the hyperthermia induced by MDMA.

Experimental design:

Forty animals were randomly divided into six experimental groups. The treatment groups were as follows:

Group I	Saline-saline
Group II	Baclofen (18 mg/kg, i.p.)
Group III	SCH-50911 (10 mg/kg, i.p.)
Group IV	MDMA (40 mg/kg, s.c.)

Group V Baclofen/ MDMA (baclofen given 45 minutes prior to MDMA)

Group VI SCH-50911/MDMA (SCH-50911 given 30 minutes prior to MDMA)

Rectal temperatures were recorded for the next 5h.

Materials:

The materials and methods used in this experiment are identical to those used in experiment three.

Methods:

Rectal temperature methods used in this study were identical to the methodology reported in experiment one. Temperatures were recorded at an environmental temperature of 23 °C.

Results:

Effect of SCH-50911 on the hyperthermia elicited by MDMA administration is shown in figure 8. MDMA resulted in a rise in body temperature starting at 2 h post injection that lasted throughout the entire period of temperature measurement. For example, rats that received MDMA alone exhibited an approximate increase of 2 °C that peaked at 3h following drug administration. Neither SCH-50911 alone nor SCH-50911 plus MDMA affected body temperature when compared to saline treated rats. Surprisingly, SCH-50911 blocked MDMA-induced hyperthermia. SCH-50911 produced a temperature decrease of approximately 1 °C 30 minutes following drug administration

in rats that received a combination of SCH-50911 and MDMA (figure 8). The body temperature of these rats closely paralleled the temperature profile of saline treated animals for the rest of the experimental period.

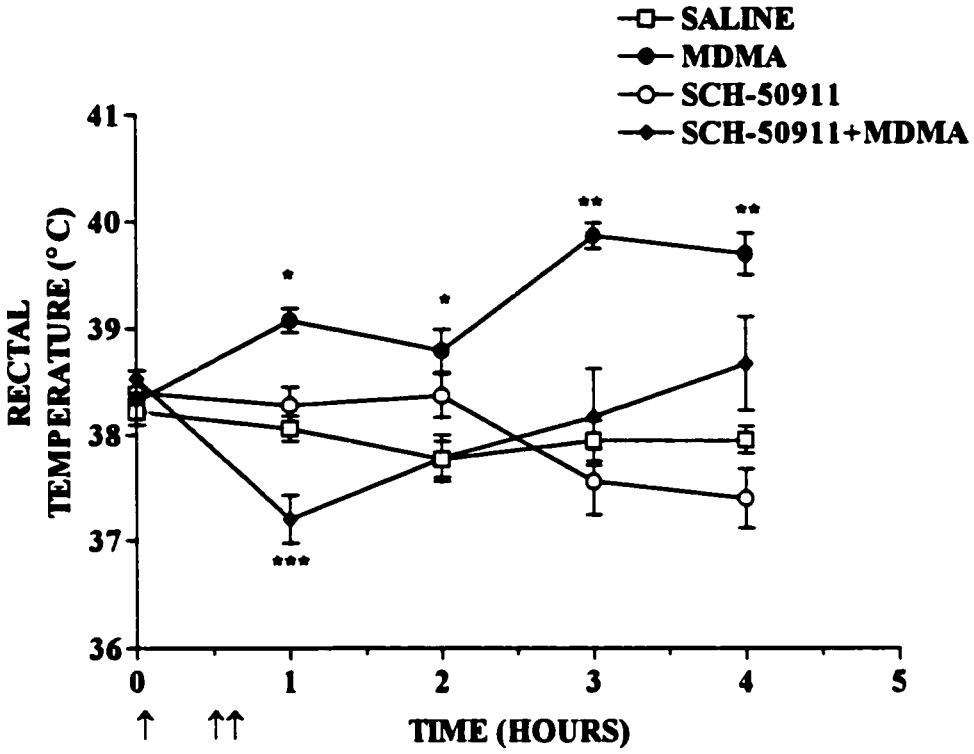

Figure 8. Effect of SCH-50911 and MDMA on temperature in rats. Rats were injected with either SCH-50911 (9 mg/kg) or saline (1 ml/kg) followed 30 minutes later by MDMA. The single arrow represents SCH-50911 injection, double arrows indicate MDMA injections. Results shown as mean ± S.E.M for 7-12 animals. *$p < 0.05$,

p < 0.01, (ANOVA, Newman Keuls post test) when compared to saline, (*p < 0.001, ANOVA, Newman Keuls post test) when compared to MDMA.

Experiment eight:

Rationale:

The neuroprotective effect of baclofen was particularly striking in brain regions that include both GABA and dopamine neurons in close synaptic apposition, namely, the striatum and the frontal cortex. Previously, Yamamoto et al. (1995) showed that decrements in GABA neurotransmisson might mediate, at least in part, the excessive release of DA mediated by MDMA. Interestingly, the abolition of the hyperthermic response following the MDMA + SCH-50911 combination raised questions regarding the basis for the thermoregulatory mechanisms altered by MDMA. Based on previous observations that attenuation of MDMA-induced hyperthermia correlates with neuroprotection, we sought to investigate the neurodegenerative effects of MDMA in the presence of MDMA.

Experimental Design:

Forty animals were randomly divided into six experimental groups. The treatment groups were as follows:

Group I	Saline-saline
Group II	Baclofen (18 mg/kg, i.p.)
Group III	SCH-50911 (10 mg/kg, i.p.)
Group IV	MDMA (40 mg/kg, s.c.)
Group V	Baclofen/ MDMA (baclofen given 45 minutes prior to MDMA)
Group VI	SCH-50911/MDMA (SCH-50911 given 30 minutes prior to MDMA)

Two weeks after MDMA administration rats were sacrificed and tissue from the striatum, hippocampus, and frontal cortex was assayed as described in experiment two.

Subjects:

Animals were handled as in experiment seven.

Materials and Methods:

The methods and materials utilized in this experiment were essentially the same as the protocol utilized in experiment six.

Results:

Effect of SCH-50911 on the neurodegenerative effects of MDMAis shown in figure 9. MDMA pretreatment produced marked reductions in serotonergic markers two weeks following drug administration. However, MDMA-induced neurotoxic effects were not potentiated by SCH-50911 as expected. To the contrary, the cortex exhibited a significant ($p < 0.01$) attenuation of MDMA-induced loss of 5-HT. Interestingly, the

striatum also exhibited a significant ($p < 0.05$) reduction of MDMA-induced loss of 5-HT markers. The partial protection that was observed in SCH-50911 pretreated rats suggests the dependency of certain brain regions on body temperature for expression of neurotoxicity. Alternatively, since the pharmacological actions of SCH-50911 are not well characterized it is possible that some previously unknown effect is mediating neuroprotection.

Figure 9. Effect of SCH-50911 (10 mg/kg) on MDMA-induced neurotoxicity two weeks following MDMA (40 mg/kg) administration. Each value is the mean ± SEM for at least 4 rats. Saline control values: A. cortex 5-HT 417.93 ± 15.61, 5-HIAA 271.87 ± 8.49 pg/mg wet wt, SERT 13.15 ± 0.54 fmole/mg wet wt; B. hippocampus 5-HT 419.74 ± 24.81, 5-HIAA 334.39 ± 21.05 pg/mg wet wt SERT 10.513 ± 0.43 fmole/mg wet wt; C. striatum 5-HT 298.33 ± 18.11, 5-HIAA 342.84 ± 19.36 pg/mg wet wt, SERT 13.15 ± 0.54 fmole/mg wet wt. Each value is the mean of 5-7 animals. Different from saline control: $^{a}p < 0.001$. Different from MDMA: $^{b}p< 0.001$, $^{c}p < 0.05$, $^{d}p< 0.01$ (ANOVA, Newman Keuls post test).

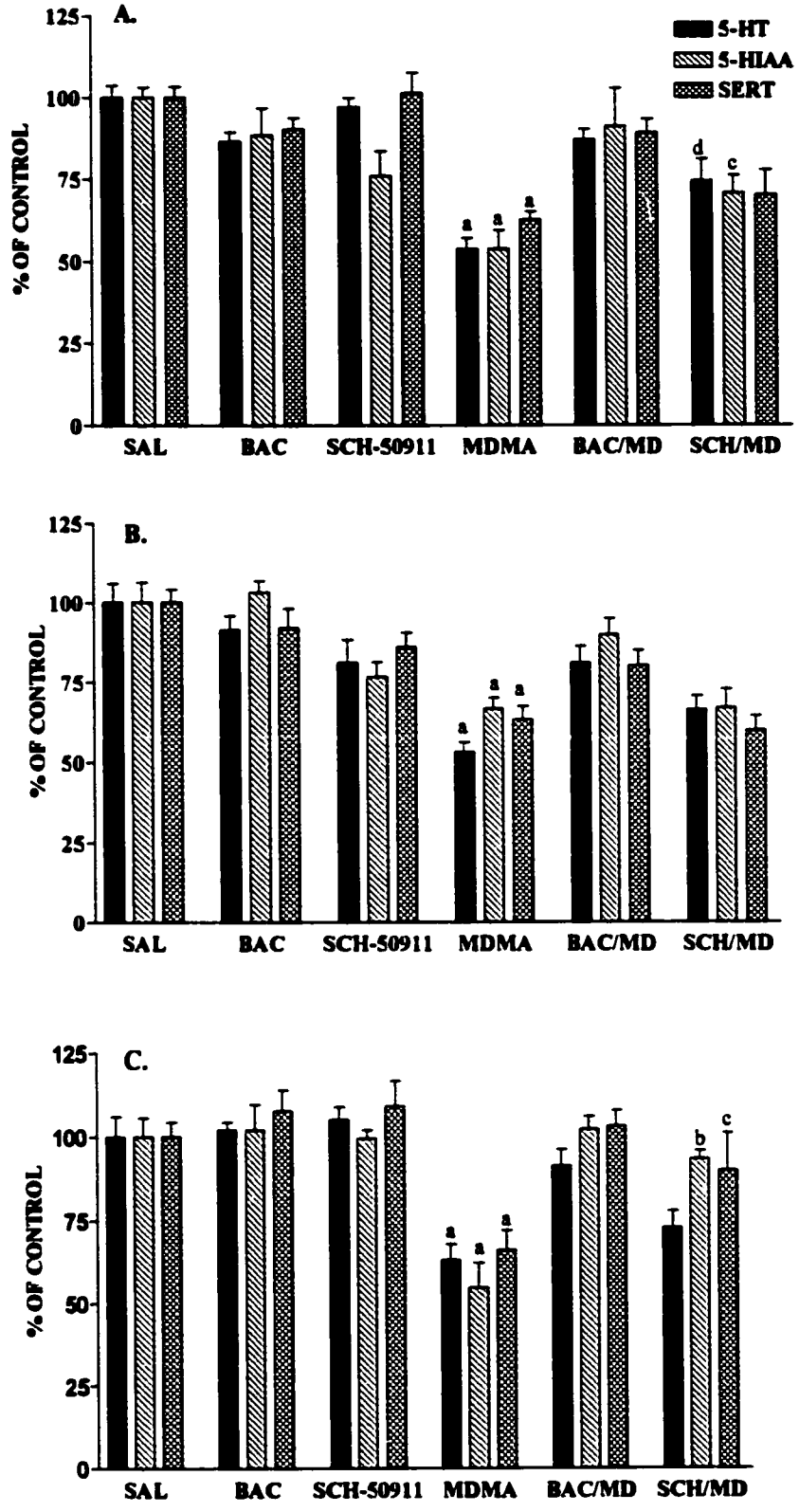
A.
B.
C.
5-HT
5-HIAA
SERT
% OF CONTROL
125
100
75
50
25
0
SAL
BAC
SCH-50911
MDMA
BAC/MD
SCH/MD

Figure 9

Role of dopamine transporter (DAT) in MDMA Neurotoxicity

Hypothesis:

MDMA-induced excessive release of DA might be due to the reversal of the DAT hence supporting the sustained increase in extracellular DA and subsequent loss of 5-HT terminals. Alternatively, DAT mediated excessive release of DA might be conducive for the expression of hyperthermia elicited by MDMA.

Experiment nine:

Rationale:

DAT has been suggested to be critically involved in the inward transport of substituted amphetamines coupled to the outward transport of DA (Liang and Rutledge, 1982). Recently, Shankaran et al. (1999) showed that DAT might be an important contributor in the MDMA-induced oxidative damage, resulting in excessive buildup of synaptic DA and ultimate loss of 5-HT terminals. Thus, the present experiment was designed to achieve maximal reduction of DAT density, in order to minimize substrate availability and carrier mediated, nonvesicular release of DA mediated by MDMA.
The lack of availability of highly selective antagonists for DAT has prompted us to utilize the more advanced and highly selective antisense (AS) technology to achieve translational arrest of DAT in a region specific manner.

Experimental Design:

Thirty two rats were randomly distributed into four different treatment groups.

Group I	Saline
Group II	a-CSF (artificial cerebrospinal fluid)
Group III	DAT- AS (antisense)
Group IV	DAT- MS (missense)

Subjects:

Adult male, Sprague-Dawley rats weighing 200-230 g were obtained from Charles River Laboratories. All animals were housed under conditions as described in experiment nine.

Materials:

The oligodeoxy nucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). [^{3}H]-mazindol was obtained from Dupont NEN; all other reagents were purchased from Sigma chemical (St. Louis, MO). Stainless steel Cannulae was purchased from Plastics one, (Roanoke, VA), and the osmotic minipump (model 1007D; 0.7ml/hr flow rate) from Alza corporation (Palo alto, CA).

Methods:

Surgical implantation of osmotic minipump and oligodeoxy administration:

The availability of the cloned sequence of the DAT facilitated the design of suitable ODNs to achieve maximal reduction of DAT expression. In the first set of experiments, 18 base pair oligodeoxynucleotides (ODNs) reported by Silvia et al. (1997) comprising Antisense-I (5'-AGA TTC AGT GGA TCC AT-3') and missense-I (5'-AGC ATT GAA CAA GCC AT-3') were utilized. The AS-ODN was targeted to the first seventeen nucleotides in the coding region of the rat DAT (erroneous sequence), and the MS-ODN was designed to have the same G-C content as the AS-ODN. In the second set of experiments, we implemented a previously published AS sequence (Simantov et al., 1996) that was shown to produce 70% reduction of DAT density in an in vitro cell culture system. Whereas the authors utilized entire sequence phophorothioation, we utilized only end capping methodology to minimize possible neurotoxic effects associated with continuous ODN infusion. Antisense-II (5'-CTT TCA GGA CAG GAC AGA-3') and missense-II (5'-TCT ATC GAG AGC GCA GAA-3') were endcapped whereby the first three and the last three bases were phophorothioated. The ODNs were purified by the manufacturer using HPLC to a purity of approximately 96-98%. The antisense-II -ODN was complementary to nucleotides 401-418 of the rat dopamine transporter (accession #:M80570), and the missense-II was designed to contain the same G-C content as the AS, but in a scrambled fashion. In the third set of experiments antisense-III-ODN (5'-GCT CTT ACT CAT GGG TAG-3') complementary to a sequence starting six bases upstream of the translational start site, and missense III-ODN (5'-TGC TCT TAC ATC AGG GAT-3') was the scrambled version of antisense-III ODN. All the three missense ODN

sequences were checked against the GENBank to ascertain that they did not match any other known sequence. The ODNs were suspended in sterile artificial cerebrospinal fluid: (NaCl 124 mM, KCl 1 mM, KH_2PO_4 1.24 mM, $MgSO_4$ 1.3 mM, $NaHCO_3$ 26 mM, $CaCl_2$ 2.4 mM, glucose 10 mM) and used for infusion experiments. Rats were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg), and a cannula (28 gauge) connected to the osmotic minipump (0.5 μl/h, seven day duration, model 1007D, ALZET minipump) through a one inch silicon tubing that was previously filled with sterile ODNs was then stereotaxically implanted into the substantia nigra pars compacta (SNc) [(A –5.8 mm, L +1.8mm, V +8.0mm from bregma (Silvia et al.,1997., Paxinos and Watson 1986). The cannula was secured in place with dental acrylic along with three stainless steel screws threaded into the skull to minimize dislodging of the cannula and the pump was inserted into the intrascapular region. A topical antibiotic was applied to the incision area to prevent infections. The ODNs were infused at a constant dose of 500 pmole/day for a period of seven days. This treatment regimen was selected based on a previously described protocol for the central administration of antisense oligonucleotides (Sylvia et al., 1997). In order to verify correct cannula placement post-experimental dye (methylene blue) injection or direct micrscopic visualization of the cannula tract was utilized.

In all the experiments the animals were placed on a palatable diet (peanut butter cookies and grapes) following the surgery to minimize lethality. The number of rats utilized was a minimum to achieve statistical significance.

Mazindol Binding:

A modified procedure of Silvia et al. (1997) was used to quantify DAT. Striata from treated animals were rapidly dissected on a cold petri dish and quickly frozen in liquid nitrogen and stored at –80 °C until the day of assay. For each experiment, a single striatum from each subject in the individual treatment groups was homogenized in 5 mL of ice-cold lysis buffer (50 mM Tris, 300 mM NaCl, 5 mM KCl; pH 7.9) and centrifuged at 36,500 x g for 10 min with an intermittent wash of the pellet with the same volume of the buffer. The pellet was resuspended in 3 ml of binding buffer in order to achieve a protein concentration of 200 µg/ml. Nonspecific binding was defined using 1 µM mazindol and 50 nM imipramine (Javitch et al., 1984). Following the addition of single high concentration (15 nM) of [^{3}H]-mazindol (K_D=18 nM), (Eich et al., 1996) incubations were started by adding 200 µl of tissue homogenates to tubes containing 1.5 ml of buffer in order give a final volume of 2.0 ml. Tubes were allowed to equilibrate on ice for 60 min before adding 5 ml of ice cold buffer and filtering through GF/B filters using a Brandel cell harvester. The tubes were subjected to two 5 ml washes with ice cold buffer and the filters were placed in plastic vials containing scintillation cocktail. The vials were allowed to stand overnight prior to counting at an efficiency of 40%.

Results:

Effects of antisense sequences on DAT binding sites in the striatum and cortex are shown in table 2.

Constant infusion of antisense-I and II via an indwelling cannula into the subtantia nigra compacta (SNc) produced only a 40% reduction in [^{3}H]-mazindol labeled DAT uptake

sites in the ipsilateral striatum. The lack of a completely complementary homology (only 76%) between AS-ODN-I and the rat mRNA for the DAT might have contributed to marginal reduction in the DAT levels in the striatum. A similar magnitude of reduction in the levels of DAT was obtained with AS-II. It is possible that lack of complete phosphorothioation of the AS ODN might have contributed to lowered levels of DAT reduction achieved following constant infusion of AS-II. Hence, we focused our attention on developing a new antisense directed toward the translational start site, because several studies have reported maximal reduction in protein expression upon selection of this region of the mRNA. Accordingly, antisense-III targeted to a region encompassing the start site produced a dramatic reduction (70%) in the DAT level in the ipsilateral striatum.

It was initially envisioned that statistical tests would be more powerful if the contralateral striatum could be used as a control tissue. The possibility of diffusion of ODN to the contralateral (SNc) side was addressed by assessing the density of [^{3}H]-mazindol labeled sites in the contralateral striatum. Unfortunately, a significant reduction (14%; $p<0.05$) in the density of DAT (AS-III) (Table 2) was evident on the contralateral side. Thus, all experiments were forced to utilize animals that received saline as controls. Additionally, we also assessed AS mediated knock down of DAT levels in the cortex to rule out any non specific effects of AS diffusion on to the adjacent VTA area.

TABLE 2. Effect of antisense oligodeoxynucleotide infusion on striatal and cortical [^{3}H]-mazindol binding sites.

Continuous infusion with DAT antisense (AS) produced significant decreases in mazindol binding sites both on the infused (I) side as well as on the untreated (C) side. However, constant infusion of missense oligonucleotide failed to produce any appreciable differences in the mazindol binding sites on both sides of the brain. Control (a-CSF-I) value for ODN-I was: 39.0 ± 0.93 fmol/g wet wt. Control (saline-I) value for ODN-II was: 42.0 ± 0.45 fmol/g wet wt.

Control (saline-I) value for ODN-III was: 32.1 ± 1.89 fmol/g wet wt.

Control (saline-C) value for ODN-III was: 42.5 ± 4.84 fmol/g wet wt.

Control (saline-I) value for ODN-III (CTX) was: 4.0 ± 0.77 fmol/g wet wt.

Each value represents the mean +/- S.E.M (N = 5-9 rats). $^a p < 0.001$, $^b p < 0.05$ vs control by ANOVA.

TREATMENTS	Experiment I (STR) B_{max} (fmol/mg wet wt.) (% remaining)	Experiment II (STR) B_{max} (fmol/mg wet wt.) (% remaining)	Experiment III (STR) B_{max} (fmol/mg wet wt.) (% remaining)	Experiment III (CTX) B_{max} (fmol/mg wet wt.) (% remaining)
SALINE (I)	ND	100 ± 1.07	100 ± 5.89	100 ± 19.625
a-CSF (I)	100 ± 2.41	112.4 ± 13.85	ND	ND
AS (I)	62 ± 2.87 [a]	60.4 ± 4.35 [a]	32.3 ± 4.46 [a]	81.06 ± 8.17
MS (I)	93.3 ± 2.23	82.1 ± 5.28	87.1 ± 4.61	77.47 ± 8.00
Contralateral (SALINE)	ND	ND	100 ± 5.0	
AS (C)	ND	ND	86.0 ± 5.0 [b]	
MS (C)	ND	ND	104.0 ± 2.32	

AS = Antisense; MS = Missense; I = Ipsilateral side; C = Contralateral side; and ND = Not done; STR = striatum; CTX = cortex

Experiment ten:

Rationale:

Attenuation of MDMA-induced increase in body temperature has been shown to afford neuroprotection (Malberg et al., 1996). The neural substrates that might be involved in the hyperthermic effects of MDMA remain elusive. Hence, the purpose of the present study was to examine whether MDMA-induced hyperthermia was altered in DAT knockdown rats.

Experimental Design:

Group I	Saline
Group II	MDMA (20 mg/kg, s.c, twice daily for 4 days)
Group III	AS/MDMA (20 mg/kg, s.c given a total of 2 doses every 12h; one week following ODN infusion)
Group IV	MS/MDMA (20 mg/kg, s.c., given a total of 2 doses every 12h; one week following ODN infusion)

Subjects:

Animals were housed, maintained, and handled as in experiment one.

Materials:

The materials used in this experiment were the same as those utilized in experiment nine.

Methods:

Rectal temperature was recorded for 6 hours following MDMA.

Results:

MDMA (20 mg/kg, s.c. b.i.d.) resulted in a hyperthermic response that persisted for at least 5 h after MDMA administration. Antisense infused animals still exhibited the increase in body temperature characteristic of MDMA (figure 10).

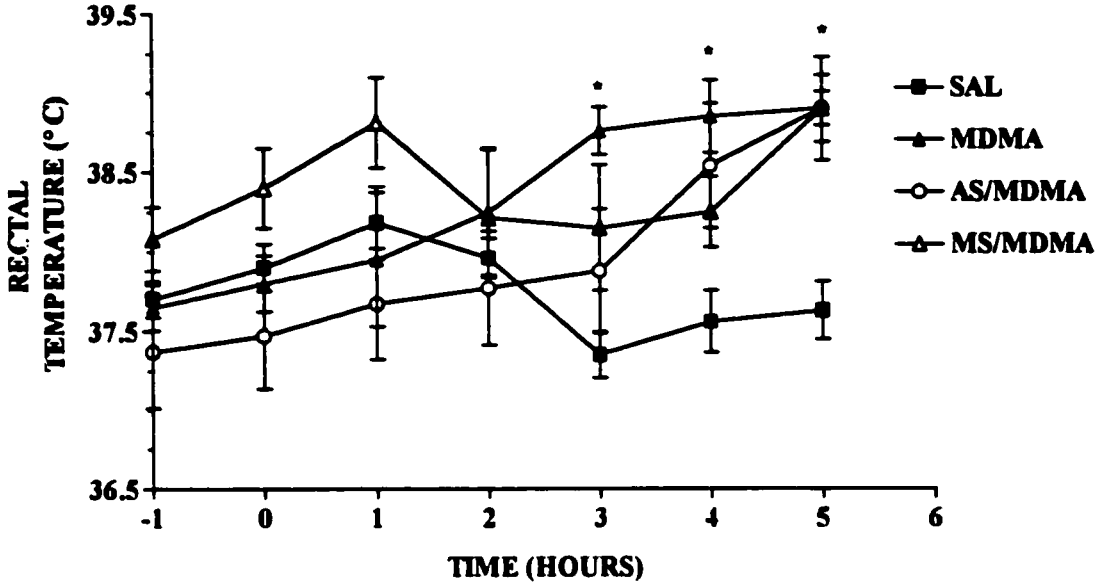

Figure 10. Rectal temperature in rats administered MDMA that previously received intranigral infusion of AS-III directed to the DAT. AS or MS were infused into the substantia nigra by an osmotic minipump for 7 days prior to MDMA (20 mg/kg, every 12h for 1 day). Results are shown as Mean ± SEM, n = 3-6. There was no significant difference in the basal temperature between groups. The group treated with MDMA alone produced a significant (*$p < 0.01$, ANOVA, Newman Keuls post test) rise in body temperature compared with the saline treated group. The AS/MDMA and MS/MDMA treatment groups were not significantly different from the MDMA treated group.

Experiment eleven:

Rationale:

Recently, it was demonstrated using microdialysis studies that MDMA produces a DA dependent increase in the formation of hydroxy radicals in the striatum, presumably by DA released through the DAT (Shankaran et al., 1999). In addition, there is strong evidence that the DAT is an important substrate in mediating the pronounced increases in extracellular DA that follow MDMA administration. Previously, Silvia et al., (1997), using an identical AS sequence demonstrated that antisense treated rats had a 32% reduction in DAT levels in the striatum. In addition, their antisense treated rats also displayed contralateral rotations following amphetamine (2 mg/kg) administration, but not after cocaine at a similar dose. Since imbalance in dopamine tone is known to induce rotational activity, unilateral AS mediated knockdown of DAT was believed to be the mechanism responsible for the altered behavioral rotational response elicited by amphetamine. Taken together, in the present experiment we sought to investigate whether 40% knockdown of the DAT with AS-I was sufficient to attenuate persistent MDMA-induced serotonergic deficits in the striatum.

Experimental Design:

Thirty-two animals were randomly allocated to six treatment groups. The experimental groups were as follows:

Group I	Saline
Group II	AS/Saline
Group III	MS/Saline
Group IV	MDMA (20 mg/kg, s.c., b.i.d. for four days)
GROUP V	AS/MDMA (one week following ODN infusion; 20 mg/kg, s.c.,b.i.d. for four days)
GROUP VI	MS/MDMA (one week following ODN infusion 20 mg/kg, s.c., b.i.d. for four days)

One week following the last injection of MDMA the animals were sacrificed for the determination of SERT and 5-HT levels.

Subjects:

Animals were housed, maintained, and handled as in experiment one

Materials and Methods:

The materials and methods utilized in this experiment are the same as those used in experiments nine and two.

Results:

Effects of prior DAT knockdown on MDMA-induced serotonergic toxicity one week after MDMA (20 mg/kg, b.i.d, s.c. for 4 days) are shown in figure 11. The AS-I sequence was utilized in neurotoxicity studies prior to realization of the lack of 100% homology of the Sylvia et al. (1996) AS-I sequence to rat DAT mRNA. MDMA alone produced a 50-60% reduction in 5-HT markers in the striatum. The [^{3}H]-paroxetine binding site density, which is considered to be an indirect marker of the structural integrity of serotonergic terminals revealed a 40-50% reduction in the striatum seven days following MDMA administration. In a parallel manner, rats that were pretreated with AS-I exhibited the same magnitude of MDMA-induced depletion of 5-HT and 5-HIAA levels in the striatum. Taken together, the results of the present study show that a 40% knockdown of the DAT is not sufficient to ameliorate the neurotoxic effects of MDMA in the striatum.

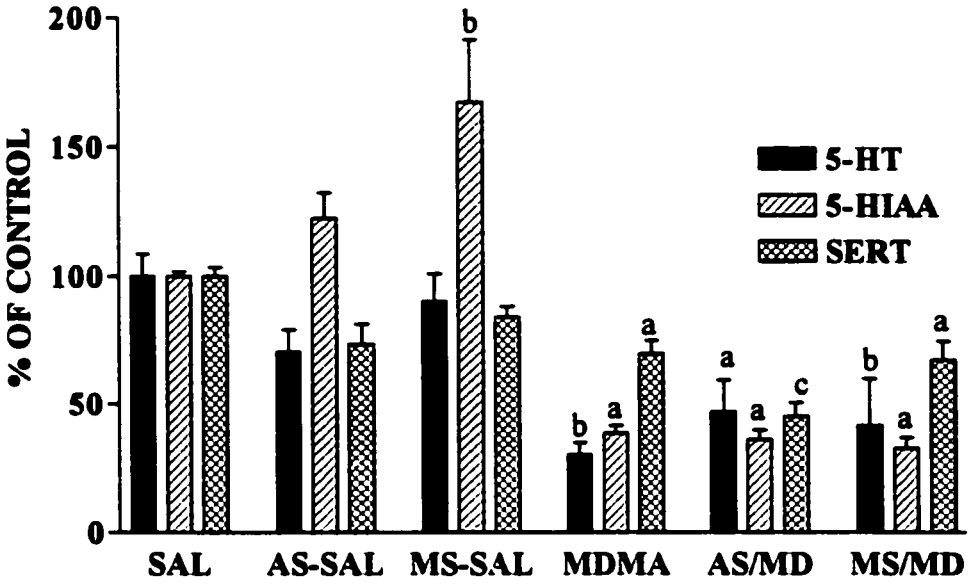

Figure 11. MDMA-induced neurotoxic response in the ipsilateral striatum after seven days of treatment with DAT AS-I ODN. Animals were subjected to constant unilateral infusion of DAT AS-I or MS ODNs (500 pmole/day) for seven days. Starting on day 8, animals were administered two injections of 20 mg/kg every twelve hours for 4 days. One week following the last dose of MDMA rats were sacrificed and the infused side was analyzed to assess the degree of MDMA-induced 5-HT neuronal loss. Saline control values for striatum: SERT 16.30 ± 0.55 fmole/mg wet wt, 5-HT 548.12 ± 23.4, 5-HIAA 1006.3 ± 17.25 pg/mg wet wt. Each value is the mean ± S.E.M for n = 4-7 animals. Significant in comparison to saline: $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ (ANOVA, Newman Keuls post test).

Experiment twelve:

Rationale:

Having demonstrated that 40% knockdown of DAT is not sufficient to attenuate MDMA-induced neurotoxicity. We tested the efficacy of AS-III in attenuating long lasting MDMA-induced reductions in 5-HT markers in the striatum. In order to evaluate the importance of region-specific AS mediated knockdown of the DAT in drug-induced neurotoxicity, we also assessed the loss of 5-HT markers induced by MDMA in other regions such as the hippocampus and the cortex.

Experimental Design:

Thirty-two animals were randomly allocated to six treatment groups. The experimental groups were as follows:

Group I Saline

Group II AS/Saline

Group III MS/Saline

Group IV MDMA (20 mg/kg, s.c., b.i.d for one day)

GROUP V AS/MDMA (one week following ODN infusion; two injections of 20 mg/kg, s.c, given every 12 h for 1 day)

GROUP VI MS/MDMA (one week following ODN infusion; two injections of 20 mg/kg, s.c, given every 12 h for 1 day)

One week following the last injection of MDMA the animals were sacrificed for the determination of SERT and 5-HT levels.

Subjects:

Animals were housed, maintained and handled as in experiment one

Materials and Methods:

The materials and methods utilized in this experiment are the same as those used in experiments two and nine.

Results:

Effects of prior DAT knockdown on MDMA-induced serotonergic toxicity one week after MDMA (20 mg/kg, b.i.d, s.c) are shown in figure-12. MDMA alone produced a 50-60% reduction in 5-HT markers in the striatum, hippocampus, and striatum. Animals that received AS-ODN prior to MDMA however, showed a dramatic reversal of MDMA-induced reduction in 5-HT, but only in the striatum. By contrast, no protection occurred in hippocampus or cortex against MDMA-induced depletion of 5-HT and 5-HIAA levels. Clearly, the attenuation of serotonergic deficits induced by MDMA only in striatum; a target region for SNc projections, suggests a role for the DAT in the MDMA-induced loss of 5-HT terminal integrity. Indeed, the results are consistent with previous data (Shankaran et al., 1999) that DAT inhibition with mazindol prior to MDMA attenuated the depleting effects of MDMA on 5-HT levels in the striatum. The absence of any significant change in MDMA-induced loss of 5-HT in missense treated rats

illustrates the specificity of AS mediated DAT knockdown on MDMA-induced serotonergic deficits. The antisense or missense sequence alone produced no significant changes in 5-HT and 5-HIAA levels.

The [^{3}H]-paroxetine binding site density, which is considered to be an indirect marker of the structural integrity of serotonergic terminals revealed a 40-50% reduction in this parameter in the forebrain regions 7 days following MDMA injection. In parallel with the above results rats that were treated with AS and MDMA had no MDMA-induced depletion of SERT density in the striatum. MDMA had the expected neurotoxic effect in cortex and hippocampus. Antisense and missense alone produced no significant changes in SERT, hence confirming the importance of the DAT in MDMA-induced persistent loss of 5-HT markers (figure 12).

Figure 12. MDMA-induced neurotoxic response after seven days of treatment with DAT AS-III ODN. Animals were subjected to constant unilateral infusion of DAT-ODNs, namely the DAT AS-III or MS ODNs (500 pmole/day) for seven days. Starting on day 8 animals were administered two injections of MDMA (20 mg/kg) every twelve hours for one day. One week following MDMA administration rats were sacrificed and the striatum from the infused side was analyzed to determine the degree of MDMA-induced 5-HT neuronal loss. Saline control values for cortex (A): SERT 12.33 ± 0.56 fmole/mg wet wt, 5-HT 230.1 ± 11.06, 5-HIAA 72.81 ± 10.04 pg/mg wet wt; hippocampus (B): 9.91 ± 0.71 fmole/mg, 5-HT 231 ± 13.2, 5-HIAA 215 ± 38.5 pg/mg wet wt; striatum (C): SERT 13.6 ± 0.68 fmole/mg wet wt, 5-HT 326.1 ± 48.60, 5-HIAA 252.0 ± 14.09 pg/mg wet wt. Each value is the mean ± S.E.M for n = 4-7 animals. Significant in comparison to saline: $^{a}p < 0.001$, $^{b}p < 0.01$; $^{c}p < 0.05$.Significant in comparison to MDMA: $^{d}p < 0.05$, $^{e}p < 0.01$, (ANOVA, Newman Keuls post test).

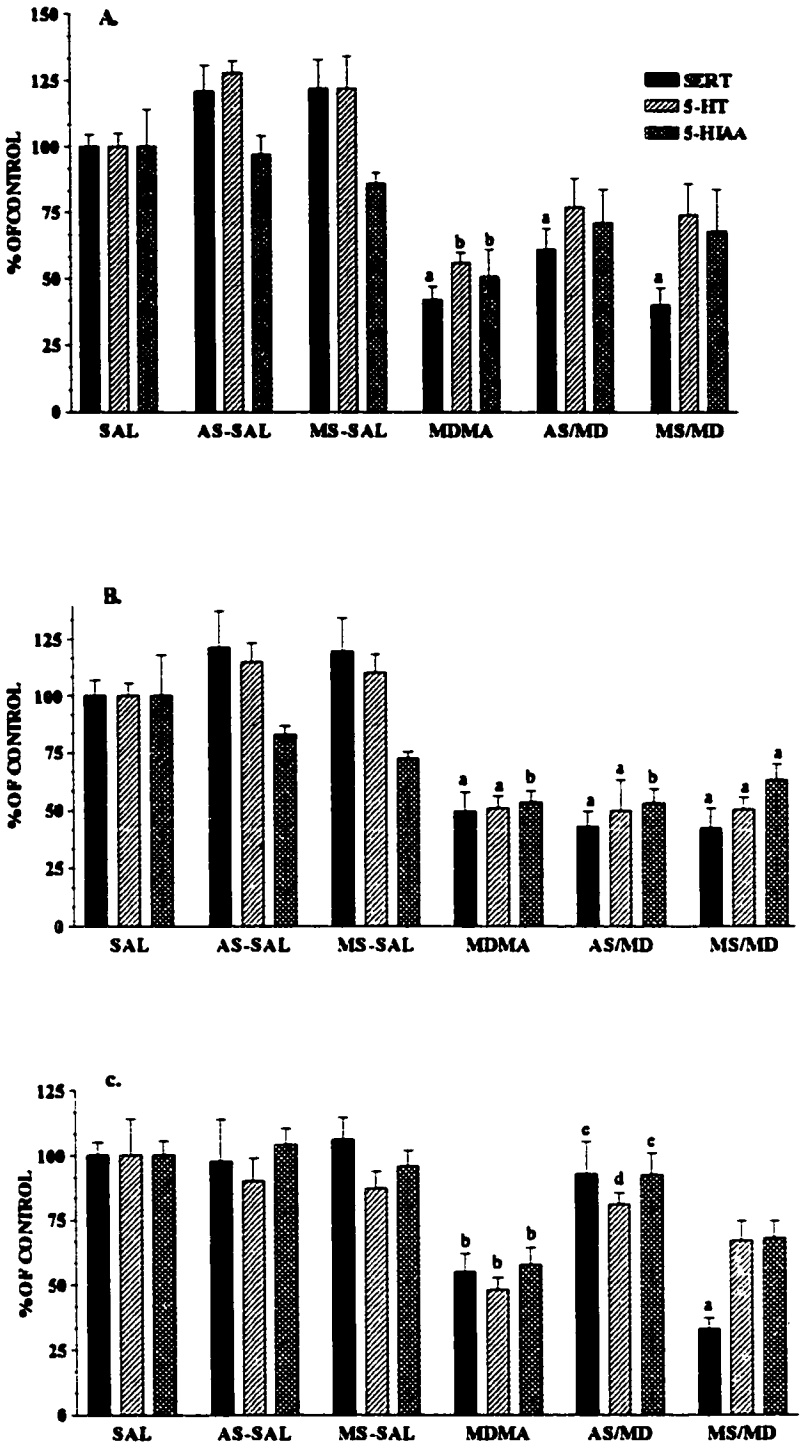
A.
150
125
100
75
50
25
0
% OF CONTROL
SERT
5-HT
5-HIAA
SAL
AS-SAL
MS-SAL
MDMA
AS/MD
MS/MD
B.
125
100
75
50
25
0
% OF CONTROL
SAL
AS-SAL
MS-SAL
MDMA
AS/MD
MS/MD
c.
125
100
75
50
25
0
% OF CONTROL
SAL
AS-SAL
MS-SAL
MDMA
AS/MD
MS/MD

Figure 12

Experiment thirteen:

Rationale:

To determine whether continuous DAT-AS infusion into the SNc produced significant reductions in dopaminergic parameters 7 days following MDMA administration or 14 days following AS-infusion, this experiment was performed to show that there was no significant effect on DA markers 7 days following the start of AS infusion, providing evidence for the integrity of dopaminergic neurons. Conversely, significant alteration of the DA markers might be reflective of a long lasting neurotoxic effect of AS or some unknown nonspecific effect on the homeostasis of dopamine neurotransmission.

Experimental Design:

Group I	Saline
Group II	AS/Saline
Group III	MS/Saline
Group IV	MDMA (20 mg/kg, s.c., b.i.d for one day)
GROUP V	AS/MDMA (one week following ODN infusion; 20 mg/kg, s.c., given every 12 h, for one day)
GROUP VI	MS/MDMA (one week following ODN infusion; 20 mg/kg, s.c., given every 12 h for one day)

Subjects:

Animals were housed, maintained and handled as in experiment one.

Materials and Methods:

The materials and biogenic amine analysis utilized in this study were the same as in experiment two.

Results:

The administration of MDMA (20 mg/kg, administered every 12h for one day) did not produce any significant change in the concentration of dopamine or its metabolite DOPAC or HVA in the striatum seven days following drug treatment (figure 13). AS or MS alone administered to animals, however, showed a trend toward reduction in DA and its metabolite levels, but the differences between groups failed to reach significance. The lack of any significant effect on striatal DA and its metabolite levels suggests that the neuroprotective effects evident in AS infused rats that received MDMA can be attributed to the specific knockdown of DAT and are not related to a nonspecific effect on dopamine terminal integrity.

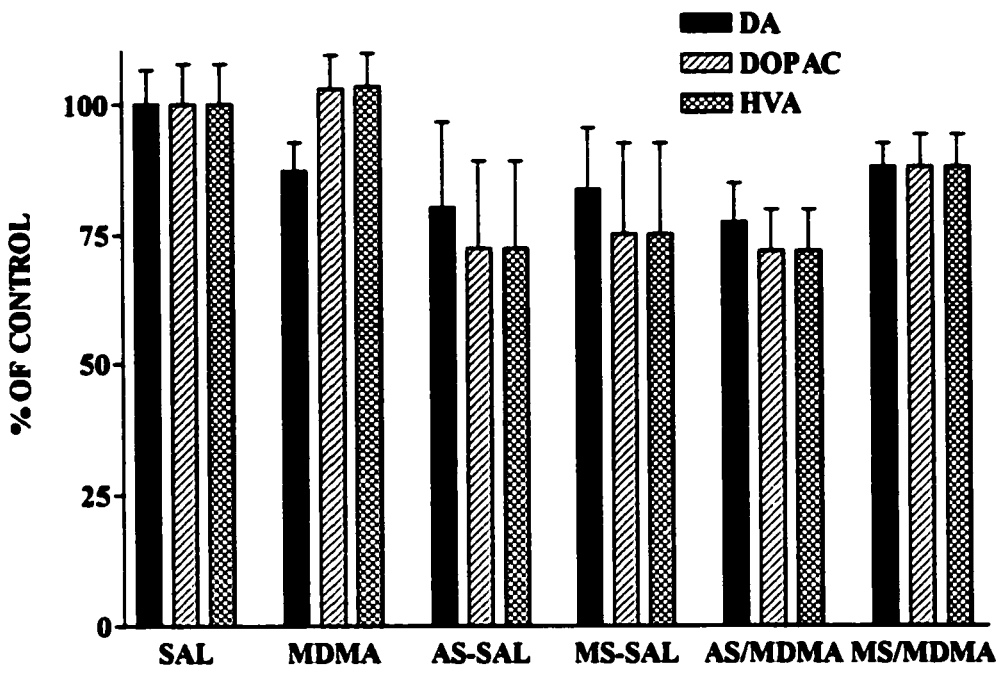

Figure 13. Effect of AS-III mediated knockdown of DAT on striatal dopaminergic parameters in rats seven days after MDMA treatment. Catecholamine levels in the striatum remained unchanged between groups. Values are presented as the mean ± S.E.M for n = 4 - 7. Saline control values for striatum were: DA 5874 ± 386.5; DOPAC 1282 ± 98.88, HVA 438 ± 8.98 pg/mg wet wt.

Role of Oxidative Stress in MDMA-induced 5-HT Terminal Degeneration

Hypothesis:

We hypothesized that MDMA-induced generation of hydroxy radicals is associated with COX-2 activation that results in a hyperthermic response, and subsequent

long-term loss of 5-HT markers. The availability of SC-236 at the time of COX-2 induction facilitated by pretreatment of SC-236 prior to MDMA might presumably lead to attenuation of the COX-2 mediated oxidative products and the associated neurotoxic events.

Experiment Fourteen:

Rationale:

Previously Colado et al. (1999d) demonstrated the close parallelism between MDMA-induced hyperthermia and generation of hydroxyradicals. Additionally, Nakayama et al. (1998) demonstrated the induction of COX-2 in the hippocampus at 2h following ischemic brain injury. In the current experiment we tested whether antagonism of COX-2 activation might attenuate MDMA-induced hyperthermia.

Experimental Design:

Thirty-two animals were randomly allocated into five treatment groups. The experimental groups were as follows:

Group I	Saline
Group II	Saline/MDMA (20 mg/kg, s.c, twice, every 12 h, for the next 24 h)
Group III	SC-236 (5 mg/kg, 30 min prior to each saline injection)/ Saline
Group IV	Methyl cellulose (3 % solution, i.p, twice, every 12 h, for the next

24 h)/ Saline

Group V SC-236/MDMA (5 mg/kg, i.p, 30 min prior to each MDMA injection)

Body temperature was recorded for the next five hours to determine whether SC-236 significantly blocked the hyperthermic response elicited by MDMA.

Subjects:

Animals were housed, maintained, and handled as mentioned previously.

Materials:

SC-236 was a generous gift from Searle Pharmaceuticals. The other materials and methods utilized in this experiment were the same as those used in experiment one.

Methods:

Rectal temperature measurements were performed in a manner identical to that described in experiment one.

Results:

MDMA resulted in an elevation of body temperature that lasted for at least 5 hours after MDMA treatment (figure 14). We observed however, that animals receiving both SC-236 and MDMA showed a significant ($p < 0.01$) reduction in MDMA-induced hyperthermia. Again, SC-236 alone produced no significant difference in body temperature when compared to saline treated animals, but only attenuated the MDMA-induced rise in body temperature. Hence, it may be inferred from this preliminary

experiment that COX-2 activation might mediate, at least in part, the MDMA-induced hyperthermia.

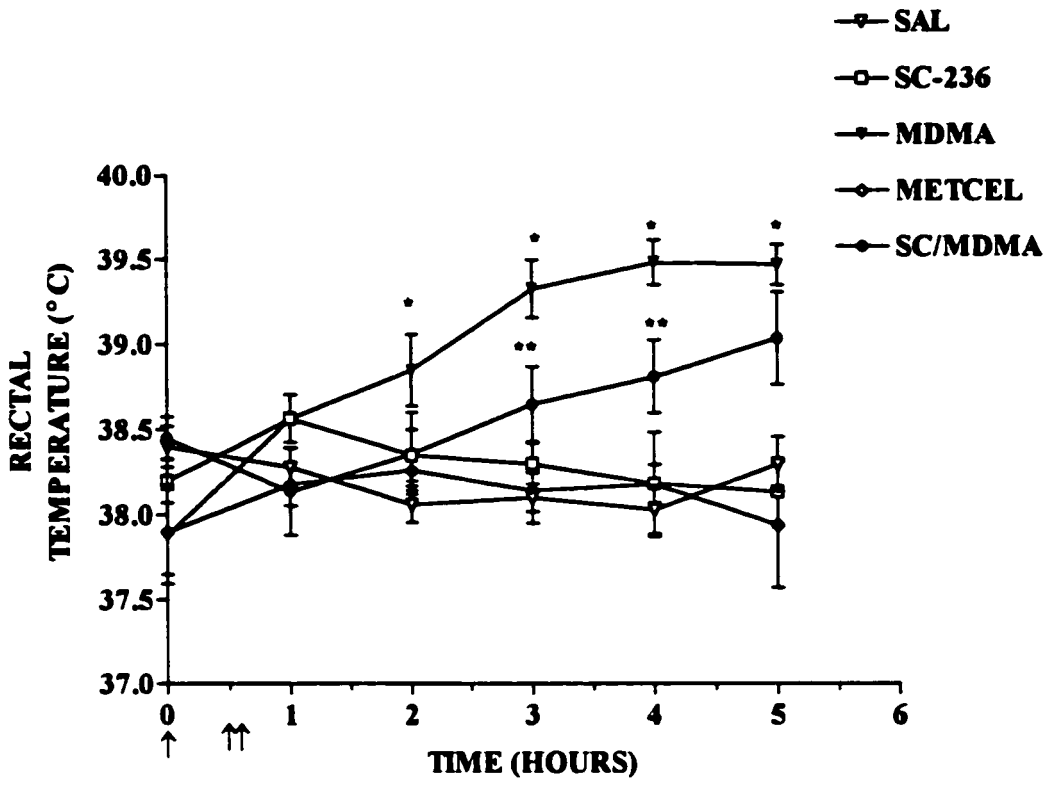

Figure 14. Effect of SC-236 on MDMA-induced hyperthermia. SC-236 (5 mg/kg, i.p.) was administered 30 minutes prior to MDMA (20 mg/kg, s.c.) and the body temperature was monitored for the next 5 h. The single arrow represents SC-236 injection; double arrows indicate MDMA injections. Temperature of MDMA treated animals was significantly different from saline treated rats (*$p < 0.001$, ANOVA, Newman Keuls post test) beginning at 2 h post MDMA injection. Results are presented as mean ± S.E.M, n =

4-7. SC-236 pretreated rats exhibited a lower (**p < 0.01, ANOVA, Newman Keuls post test) core body temperature when compared to MDMA treated rats over time, although the core temperature was still higher than saline treated rats. The solvent methyl cellulose that was used to resuspend SC-236 had no significant effect on body temperature when compared to saline treated rats.

Experiment fifteen:

Rationale:

We speculated that since SC-236 significantly attenuated the hyperthermic response elicited by MDMA, it is reasonable to expect neuroprotection at least, in certain brain regions namely the cerebral cortex, and hippocampus that have been previously shown to exhibit high levels of COX-2 expression following exposure to neuroinflammatory or neurodegenerative events (Nogawa et al 1998).

Experimental Design:

Thirty-two animals were randomly allocated into three treatment groups. The experimental groups were as follows:

Group I	Saline
Group II	Saline/MDMA (20 mg/kg, s.c, twice, every 12 h, for the next 24 h)
Group III	SC-236 (5 mg/kg, 30 min prior to each saline injection)/ Saline
Group IV	Methyl cellulose (3% solution, i.p, twice, every 12 h, for the next

24 h)/ Saline

Group V SC-236/MDMA (5 mg/kg, i.p, 30 min prior to each MDMA injection)

Seven days following MDMA administration, the animals were decapitated and the striatum, hippocampus, and frontal cortex were assayed for 5-HT markers.

Subjects:

Animals were housed, maintained, and handled as in experiment eleven.

Materials and Methods:

The materials utilized in this experiment were the same as those utilized in the previous experiment and the rectal temperature measurements were performed in a manner identical to that described in experiment one.

Results:

The effect of SC-236 on the long term depletion of 5-HT and 5-HIAA produced in the cortex 7 days after MDMA administration is shown in Figure 15. MDMA alone produced dramatic reductions (40-60%) in the concentrations of 5-HT markers in all the regions assayed for neurotoxicity. SC-236 given thirty minutes before MDMA, however, was effective in attenuating the long term reductions in cortical 5-HT and 5-HIAA. In contrast, there was no significant neuroprotection against MDMA-induced neurotoxicity

in the hippocampus or the striatum. Rats pretreated with SC-236, however showed a trend toward a slight increase in 5-HT markers, although the differences were not significant in comparison to saline treatment. The results suggest regional difference in the susceptibility to COX-2 activation and subsequent neurotoxicity.

Figure 15. Effect of MDMA (20 mg/kg, b.i.d., s.c. for one day) and SC-236 (5 mg/kg administered 30 minutes before MDMA) on MDMA-induced neurotoxic effects one week after dug administration. Each value is the mean ± SEM for 4 - 7 rats. Saline control values: cortex (Panel A) 5-HT 223 ± 34.2, 5-HIAA 85.8 ± 8.92 pg/mg wet wt, SERT 14.7 ± 0.91 fmole/g wet wt; hippocampus (Panel B) 5-HT 200.0 ± 16.04, 5-HIAA 148.30 ± 6.41 pg/mg wet wt SERT 13.3 ± 0.548 fmole/g wet wt; striatum (Panel C) 5-HT 337 ± 6.37, 5-HIAA 168 ± 9.72 pg/mg wet wt, SERT 14.4 ± 1.39 fmole/g wet wt. Different from saline control: $^{a}p < 0.01$, $^{b}p < 0.001$. Different from MDMA: $^{c}p < 0.05$, $^{d}p < 0.01$ (ANOVA, Newman Keuls post test).

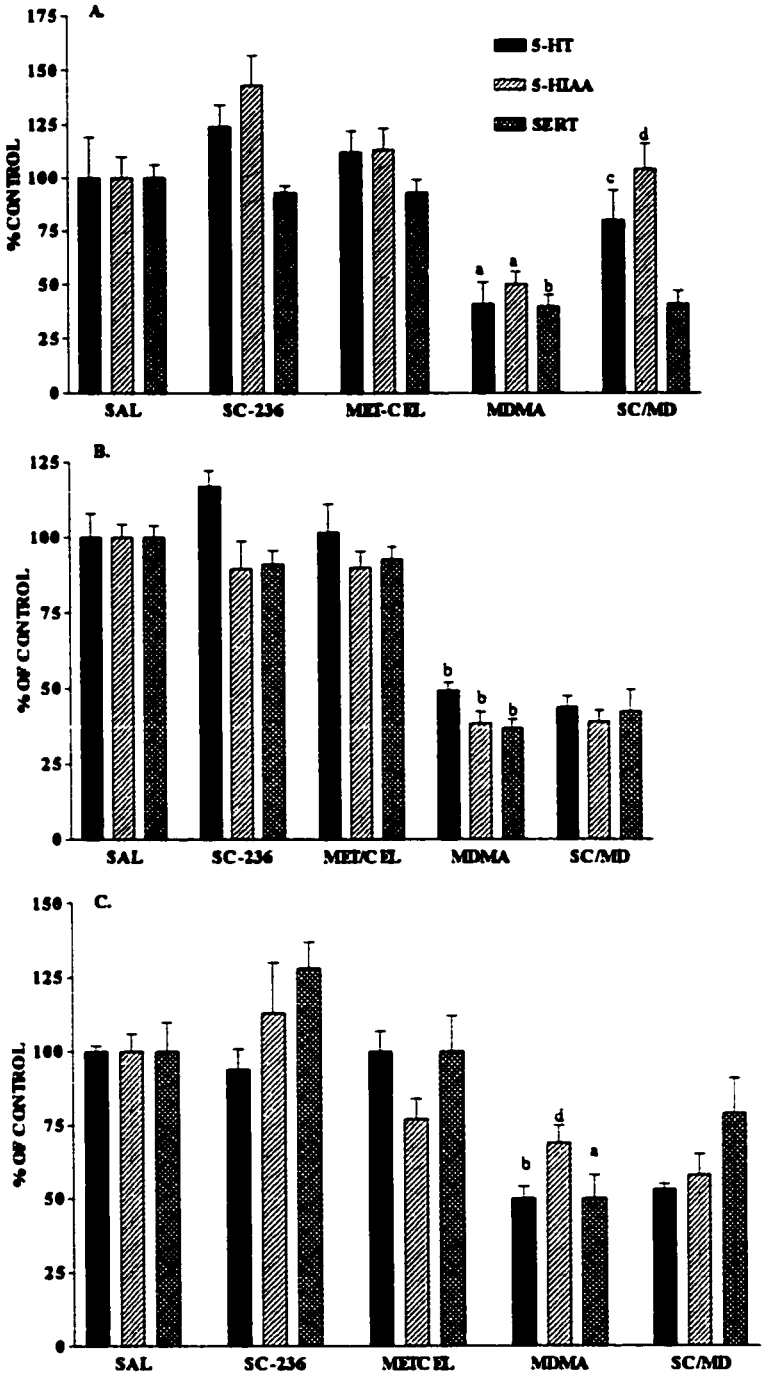
A.
5-HT
5-HIAA
SERT
% CONTROL
175
150
125
100
75
50
25
0
SAL
SC-236
MET-CEL
MDMA
SC/MD
B.
% OF CONTROL
125
100
75
50
25
0
SAL
SC-236
MET/CEL
MDMA
SC/MD
C.
% OF CONTROL
150
125
100
75
50
25
0
SAL
SC-236
MET/CEL
MDMA
SC/MD

Figure 15

Experiment sixteen:

Rationale:

SC-236 is a COX-2 inhibitor with a very long plasma half life (5 days) (Penning et al., 1997). Therefore, we sought to investigate as to whether, SC-236 mediated COX-2 antagonism produced any significant alterations in dopaminergic parameters 7 days following drug administration.

Experimental Design:

Thirty-two animals were randomly allocated into three treatment groups. The experimental groups were as follows:

Group I	Saline
Group II	Saline/MDMA (20 mg/kg, s.c, twice, every 12 h, for the next 24 h)
Group III	SC-236 (5 mg/kg, 30 min prior to each saline injection)/ Saline
Group IV	Methyl cellulose (3% solution, i.p, twice, every 12 h, for the next 24 h)/ Saline
Group V	SC-236/MDMA (5 mg/kg, i.p, 30 min prior to each MDMA injection)

Subjects:

Animals were housed, maintained and handled as in experiment one.

Materials and Methods:

The materials utilized in this study came from the same sources as noted in experiment fifteen. The biogenic amine analysis utilized in this study was the same as in experiment two.

Results:

The administration of MDMA (20 mg/kg, s.c.) did not produce any significant change in the concentration of dopamine or its metabolite DOPAC in the striatum (figure 16). In a similar manner, dopaminergic parameters remained unaffected in rats that received a combination of SC-236 and MDMA. Likewise, SC-236, or methyl cellulose, alone produced no significant changes in DA markers.

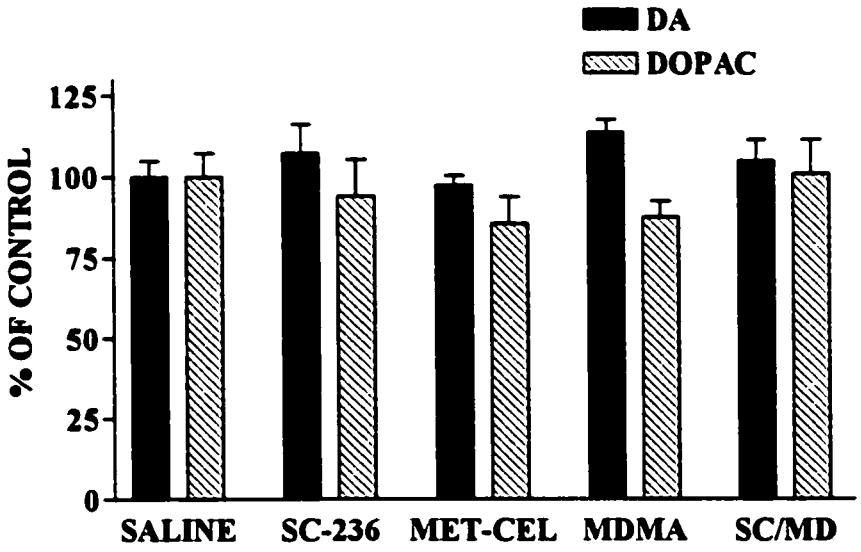

Figure 16. Effect of SC-236 inhibition of COX-2 on striatal dopaminergic markers in rats seven days after MDMA treatment. Catecholamine levels in the striatum remained

unchanged between groups. Values are presented as the mean ± S.E.M for N = 4 - 7. Saline control values for striatum were: DA 5115 ± 248; DOPAC 1025 ± 79.23 pg/mg wet wt.

DISCUSSION

The five different studies outlined in this thesis highlight the critical roles of DAT, GABA, DA, and COX-2 activation in MDMA-induced serotonergic neurotoxicity. MDMA-induced 5-HT terminal loss can be partially attributed to a process that results from decrements in GABA neurotransmission leading to a reciprocal increase in DA neurotransmission via a 5-HT_{2A} receptor-mediated mechanism. Therefore, the study design was primarily aimed at understanding the influences of increased GABAergic transmission, reduced levels of functional DAT, and inhibition of COX-2 activity in MDMA-induced dysregulation of body temperature and loss of 5-HT terminal integrity. Thus, the objectives and the outcomes of our findings are summarized below.

1) If diminished extracellular GABA levels are partly responsible for the long term reductions in 5-HT markers mediated by MDMA, the obvious corollary would be that enhancement of extracellular GABA levels would circumvent the inhibitory effect of MDMA on GABA neurotransmission and prevent the loss of 5-HT markers. Indeed, the current study demonstrates that potentiation of GABAergic transmission prior to MDMA treatment attenuates MDMA-induced 5-HT deficits.

2) To investigate further the mechanism of neuroprotection afforded by GVG, we probed the role of GABA-B receptors in MDMA-induced neurotoxicity. GABA-B receptor activation exerts a negative modulatory effect on dopaminergic transmission in nigrostriatal and mesolimbic areas of the brain (Santiago et al., 1992). Hence, we anticipated that this study would shed light on the functional significance of GABA-B receptor stimulation on excessive dopaminergic transmission facilitated by MDMA. In fact, the results of the present study demonstrate that GABA-B activation using baclofen, not only provides partial protection against MDMA-induced excessive dopaminergic transmission but also against the long term reduction of 5-HT markers mediated by MDMA. Additionally, baclofen pretreatment also abolished MDMA-induced hyperthermia. Taken together, the current study demonstrates diverse pharmacological effects of baclofen in blocking the long-term reductions in 5-HT markers mediated by MDMA and provides further evidence to support the involvement of GABAergic pathways in the neurotoxic process.

3) To examine further the neural substrates that mediate MDMA-induced excessive dopaminergic transmission, we focused our attention on elucidating the role of the DAT in MDMA neurotoxicity. Previously, the role of the DAT in MDMA induced neurotoxicity has been inferred using pharmacological interventions (Stone et al., 1989; Shankaran et al., 1999). The recent availability of antisense (AS) oligonucleotide technology provides the unique advantage of testing directly the dependence of MDMA induced neurotoxic effects on the DAT. In the present

study, AS mediated knock-down of the DAT prior to MDMA blocked the detrimental effects of MDMA on striatal 5-HT terminal integrity without any appreciable effect on the hyperthermic response elicited by MDMA. Thus, this study demonstrates that the DAT is probably an obligatory component for the expression of MDMA toxicity *in vivo*. The increase in body temperature mediated by MDMA probably occurs through a different pathway.

4) If MDMA-induced hyperthermia and neuronal degeneration is mediated via COX-2 dependent mechanisms, then antagonism of COX-2 enzyme, well-known for its activation during neuronal injury, should offset the neurotoxic potential of MDMA. Indeed, the results of the current study demonstrate that COX-2 mediated reaction products might be involved in MDMA-induced neurotoxicity in a region specific manner.

These findings are more fully detailed below under individual sections to highlight the significance of each process in MDMA-induced neurotoxicity.

Role of Increased Extracellular GABA

According to the hypothesis that MDMA-induced neurotoxicity involves GABAergic pathways, MDMA-induced 5-HT release in the striatum and/or substantia nigra is believed to inhibit striatonigral GABA release with a concomitant disinhibition of the nigrostriatal dopaminergic system via a 5-$HT_{2A/2C}$ receptor mediated mechanism (Yamamoto et al., 1995). Furthermore, these workers blocked the inhibitory effect of MDMA-induced 5-HT release on GABA function in the striatum by local infusion of the

5-HT_2 receptor antagonist ritanserin, hence confirming a role for the 5-HT_2 receptor in the GABAergic impairments produced by MDMA. In the current study, MDMA alone induced acute hyperthermia and marked depletion of the serotonergic markers, 5-HT, 5-HIAA, and SERT density in the frontal cortex, hippocampus, and striatum of rats seven days after the last dose of MDMA (Battaglia et al., 1987; Johnson et al., 1991; Stone et al., 1987). However, in rats that were pretreated with γ-vinyl-GABA (GVG), an irreversible suicide inhibitor of GABA-transaminase (GABA-T) prior to MDMA, substantial reduction in the acute hyperthermic response and decreased long-term loss of 5-HT markers was evident. Therefore, the current study provides further support for the GABAergic hypothesis in MDMA-induced neurotoxicity, whereby decreases in extracellular GABA may contribute, at least in part, to the neurotoxic effects elicited by MDMA.

GVG is known to inhibit the degradation of GABA via irreversible suicide inhibition of neuronal GABA-T. This action increases GABAergic transmission by increasing the presynaptic availability and subsequent release of GABA (Qume et al., 1987). Moreover, GVG has been shown to increase extracellular levels of GABA in the striatum (Dewey 1997), and increase potassium stimulated GABA release from cerebral cortex synaptosomes (Abdul Ghani et al., 1981), as well as from hippocampal slices (Qume and Fowler 1997). GABA is known to modulate the excitability of dopamine neurons. Therefore, GVG might be expected to reduce tonic extracellular DA levels. Indeed, previous studies have shown that GVG pretreatment inhibits basal as well as cocaine-induced increases in locomotor activity and extracellular DA levels in the striatum and in the nucleus accumbens (Dewey et al., 1997; Morgan and Dewey 1997).

Furthermore, Gerasimov et al., (1999) using microdialysis studies, recently, showed that GVG diminished methamphetamine- and heroin-induced increases in extracellular levels of DA in the nucleus accumbens of freely moving rats. Taken together, these studies demonstrate that, irrespective of the mechanism of DA release mediated by psychomotor stimulants, GVG was effective in antagonizing the serotonergic neurotoxic response produced by MDMA.

In an attempt to evaluate the role of GABAergic impairments in MDMA-induced neurotoxicity, we utilized a dosing regimen that has been previously shown to produce maximal elevation of striatal GABA content. In fact, the longer duration (24 h) pretreatment strategy was chosen based on previous studies demonstrating that GVG led to a 4-6 fold increase in GABA concentration in the brain that lasted for at least 48-72 h (Sayin et al., 1995; Jung et al., 1977; Qume and Fowler, 1996). This observation is consistent with the drug's irreversible effect on GABA-T, requiring de novo synthesis of new enzyme. On the other hand, the acute dosing regimen that was employed by Dewey et al., (1997), showed a significant blockade of cocaine induced release of DA in the striatum of rats that were given GVG only 120 minutes prior to cocaine exposure. In the present study we observed neuroprotection with both dosing regimens (acute as well as long term dosing). Hence, the pretreatment time is not a critical factor for the ability of GVG to attenuate MDMA-induced long-term loss of 5-HT markers and hyperthermia. GVG also acutely inhibits GABA reuptake (Christensen et al., 1991; Jolkonen et al., 1992), therefore increases in extracellular levels of GABA mediated by GVG might have contributed to the inhibitory effect on MDMA-induced neurotoxicity.

MDMA has been shown to promote dramatic acute release of dopamine (Nash and Brodkin., 1991; Yamamoto et al., 1995; Schmidt et al., 1992) via both a carrier-mediated (Stone et al., 1988; Shankaran et al., 1999) and impulse dependent process (Gudelsky and Nash 1996; Yamamoto et al., 1995). MDMA-induced neurotoxicity may be mediated through a dopamine dependent oxidative damage to 5-HT axon terminals, attributed to access of excessive extracellular DA to the interior of the 5-HT terminal. This presumably occurs via a 5-HT carrier mediated mechanism (Colado et al., 1995; Sprague et al., 1995). The requirement of DA in the MDMA neurotoxicity mechanism has been previously supported by the following studies: 1) depleting dopamine stores with α-methyl-p-tyrosine was shown to attenuate both DA release in the striatum and the long term serotonergic deficits in the striatum, hippocampus, and cortex (Stone et al., 1988). 2) Decreasing catecholamine stores with monofluoromethyl-DOPA, a decarboxylase inhibitor, attenuated the serotonergic decrements induced by MDMA (Schmidt et al., 1990). 3) L-DOPA administration potentiated MDMA-induced 5-HT depletion (Schmidt et al 1990). Taken together, the data are supportive of a view that excessive extracellular DA, under certain circumstances, may be detrimental to the normal functioning of 5-HT neurons. Because a role for GABA in modulating DA function is well established, the current data raise the possibility that elevation of extracellular GABA levels prior to MDMA might have reinstated the normal inhibitory negative feedback inhibition on dopaminergic neurotransmission, at least in the regions where dopaminergic neuron terminals are negatively regulated by the GABAergic neurons, namely, the cortex and the striatum (Santiago et al., 1992). Thus, a GABA-

mediated reduction in extracellular DA release might have contributed to the neuroprotective effects of MDMA.

There is also compelling evidence to support the importance of hyperthermia in the neurodegenerative effects of MDMA. The magnitude of MDMA-induced reductions in 5-HT markers can be reduced by lowering body temperature or by pharmacological interventions that attenuate the hyperthermic response. Recently, the effects of several types of GABAergic agents with diverse pharmacological actions (Colado et al., 1997; Colado et al., 1998; Colado et al., 1999) were investigated for their neuroprotective effects on MDMA-induced hyperthermia, generation of hydroxy radicals, and subsequent long term loss of 5-HT markers. The authors demonstrated that clormethiazole's inhibitory effect on MDMA-induced generation of oxidative markers in the rat was abolished by elevation of body temperature, hence leading them to conclude that hypothermia might be an important factor underlying the neuroprotection mechanism conferred by chlormethiazole. Likewise, pentobarbitone's neuroprotective action against the neurodegenerative effects of MDMA in rats was reversed upon elevation of core temperature (Colado et al., 1999). Furthermore, several other agents with diverse pharmacological effects were shown to be neuroprotective by virtue of their ability to induce profound hypothermia (Farfel and Seiden, 1995; Malberg et al., 1996; Taraska and Finnegan, 1997). The results of the current study are indeed consistent with previous studies highlighting the importance of hypothermia in attenuating MDMA-induced long term deficits. The fact that hyperthermia potentiates free radical generation (Globus et al., 1995; Kil et al., 1996) is supportive of the view that MDMA-induced acute hyperthermia may be related to the formation of free radicals, perhaps through DA

oxidation (Sprague et al., 1995; Colado et al., 1999) or formation of oxidative metabolites of MDMA (Hiramatsu et al., 1990; Colado et al., 1990). Thus, hypothermia elicited by GVG might also be an important attribute of its neuroprotective efficacy against MDMA-induced long-term 5-HT axonal loss.

In conclusion, the data in this thesis provide evidence that the neuroprotective effect of GVG may be due to its ability to increase extracellular GABA, either by acute release or by inhibition of its catabolic breakdown. Although the exact mechanism of GABA release mediated by GVG is currently unknown, either leakage of increased intracellular GABA from neurones and glia or impulse dependent release might contribute to inhibition of excitatory neurotransmission (Fowler and Qume 1997). In particular, the current data also raise the possibility that indirect agonists such as GVG might counteract MDMA-induced release of DA through a GABA-B receptor mediated mechanism. Nonetheless, the role of GVG-induced hypothermia in reducing MDMA induced generation of ROS cannot be excluded. Taken together, the current data demonstrate the feasibility of utilizing agents that enhance GABAergic transmission to attenuate MDMA-induced neurotoxicity and hyperthermia.

Role of GABA-B Receptors

There is now substantial evidence that combined treatment of MDMA with GABAmimetics reduces the serotonergic damage produced by MDMA. In addition, GABAergic agents have been shown to produce pronounced hypothermia, even after MDMA, which alone induces marked hyperthermia (Colado et al 1998a, Colado et al

1994). In our previous study, we demonstrated that enhancement of extracellular GABA levels by direct release or by preventing the neurotransmitter breakdown by GABA-T ameliorated the neurotoxic effects of MDMA. Hence, in an attempt to investigate further the neurochemcial mechanism underlying GVG's neuroprotective action we probed the role of the GABA-B receptor in MDMA-induced neurotoxicity.

GABA, an inhibitory neurotransmitter in the CNS, is believed to activate three distinct classes of CNS receptors: GABA-A, GABA-B, and GABA-C subtypes (Bowery, 1993). Local as well as systemic administration of a GABA-B receptor agonist produces a decrease in firing rate and burst firing activity of DA-containing midbrain neurons (Erhardt et al., 1998, 1999). In general, GABA-B receptors modulate synaptic transmission, depending on their location and identity. Presynaptic GABA-B receptors are believed to inhibit neurotransmitter release through a reduction of the presynaptic Ca^{2+} influx, while postsynaptic GABA-B receptors contribute to hyperpolarization through an increase in K^{+} conductance (Bowery et al., 1980). Presynaptic localization of GABA-B receptor on DA terminals was further confirmed by Arias-Montano et al., (1991) who found GABA-B receptor mediated inhibition of calcium and stimulus dependent tyrosine hydroxylase activity in striatal slices from rat, an effect antagonized by the GABA-B antagonist, phaclofen hence confirming a role for a GABA-B receptor mechanism in regulating dopaminergic neurotransmission. Based on the postulated GABAergic involvement in MDMA-induced neurotoxicity, through inhibition of the striatonigral GABAergic system and consequent disinhibition of substantia nigra (SN) DA neurons in the striatum (Yamamoto et al., 1995), we hypothesized that activation of GABA-B receptors should antagonize MDMA's inhibitory effect on GABA release and

thereby compromise the ability of MDMA to increase extracellular DA levels. Indeed, the current data demonstrate that prior administration of the GABA-B receptor agonist, baclofen produces partial protection against MDMA-induced acute as well as long term alterations of monoaminergic markers. In addition, complete blockade of MDMA-induced hyperthermia was evident following baclofen pretreatment.

The present results show that MDMA alone produced pronounced reductions (50-60%) in 5-HT, 5-HIAA, and SERT in the frontal cortex, hippocampus, and striatum 2 weeks following the last dose of MDMA (figure 4). In rats that received baclofen (18 mg/kg, i.p.) 45 minutes prior to MDMA, however, significant attenuation of MDMA-induced long term reductions were evident. The current data supplement our previous observation with GVG that enhancing GABAergic transmission leads to preservation of 5-HT terminal integrity. In addition, the current results are also consistent with previous observations that GABAmimetics antagonize MDMA-induced 5-HT deficits in all the regions that are known to be vulnerable to the neurotoxic effects of MDMA (Colado et al., 1999a, 1999b, 1997). Possible mechanisms underlying baclofen's neuroprotective effect could be: 1) Acute modulation of MDMA-induced increases in dopaminergic transmission and, 2) Attenuation of MDMA-induced hyperthermia. The partial reduction in MDMA-induced acute serotonergic deficits might be attributed to the predominant role of SERT in the MDMA-induced acute release of 5-HT. Therefore, as mentioned previously, baclofen, attenuates only impulse dependent release of DA (Santiago et al., 1993) and 5-HT in the cortex (Bowery et al., 1980) and striatum and therefore, the current results suggests that baclofen mediated hyperpolarization plays only a minor role in the MDMA-induced acute depletion of monoamines.

As noted in the previous section, in recent years there has been substantial evidence that agents that are protective in other neurodegenerative states (e.g. ischemia) may attribute this protection to their inherent ability to decrease elevation in body temperature and subsequent generation of free radicals (Globus et al., 1995; Kil et al., 1996). Additionally, the degree of ROS-mediated neurotoxicity is positively correlated with environmental temperature. For example, when rats were placed in an ambient temperature of 15°C, dramatic reductions in MDMA-induced hyperthermia and long term reduction in the tissue content of 5-HT and 5-HIAA were observed (Malberg et al., 1996). Likewise, a similar pattern of neuroprotection was observed in rats that were kept at an ambient temperature of 15-20 °C. Conversely, administration of MDMA at an elevated environmental temperature (32 °C) was found to exacerbate the neurotoxicity. The increase or decrease in body temperature upon alteration of the environmental temperature indicates that MDMA administration may compromise thermoregulatory mechanisms. Clearly, temperature plays a major role in the induction and the protection against MDMA-induced neurotoxicity. In an attempt to determine the effect of coadministration of MDMA and baclofen on MDMA-induced hyperthermia, the current study demonstrates that baclofen pretreatment attenuated MDMA-induced hyperthermia by lowering the body temperature by ~2 °C in comparison to saline treated rats. Interestingly, considerable interindividual differences in the ability to attenuate MDMA-induced hyperthermia were evident: a subpopulation of rats failed to exhibit hypothermia following baclofen. This observation is consistent with previous studies (Waldmeier 1991) whereby baclofen administration produced variable effects on DOPA accumulation in the striatum, depending on the route of drug administration.

The importance of lowered body temperature on amphetamine-induced DA release was shown in a study by Bowyer et al. (1993), who demonstrated that significant attenuation of methamphetamine-induced DA release in the striatum was achieved when the animals were tested in a cooler environment. Consistent with this observation, reserpine, known for the drug's ability to deplete vesicular catecholamine and indoleamine stores, was shown to reduce body temperature to an average of 31.6 °C along with reduced MDMA-induced DA release. (Sabol et al., 1998). Reinstating the body temperature to normal in rats receiving a combination of reserpine and MDMA was expected to reverse the antagonistic effects of reserpine on MDMA-induced DA release. To the contrary, only an intermediate level of DA release that was not significantly different from rats kept at normal temperature was observed. The lack of complete reinstatement of MDMA-induced DA release upon heating argues against a role for hypothermia as the sole contributor of reserpine's effect on MDMA-induced DA release. Nevertheless, although hypothermia may play an important role in reducing the magnitude of ROS generation, hypothermia per se may not be the sole contributor for the neuroprotection afforded by baclofen.

Based on evidence that baclofen acutely exerts a negative inhibitory effect on dopaminergic transmission we investigated the drug's effects on MDMA-induced potentiation of monoaminergic transmission 3 h after drug administration. MDMA alone produced increased brain DA content, with reciprocal decreases in 5-HT, 5-HIAA, and DOPAC in the striatum. Baclofen pretreatment acutely blocked the increase in tissue DA without any appreciable effect on indoleamine content in the striatum. There is convincing evidence that MDMA, similar to amphetamine, enhances DA efflux from the

newly synthesized non-vesicular DA pool. For example, blockade of the MDMA-induced increase in DA synthesis using either a tyrosine hydroxylase inhibitor such as α-methyl-para-tyrosine (Schmidt et al., 1992; Brodkin et al., 1992) or the 5-HT_2 receptor antagonist MDL 100,907 (Schmidt et al., 1992; Schmidt et al., 1994) was accompanied by dramatic acute reductions in MDMA-induced efflux of DA in the striatum, as well as blockade of MDMA-induced long-term serotonergic deficits in forebrain regions. Since impulse-dependent release has also been shown to be a significant part of MDMA-induced neuronal efflux of DA (Nash and Gudelsky, 1996; Yamamoto et al., 1995), the possibility exists that dopamine terminal hyperpolarization via baclofen might have blocked, at least in part, MDMA-induced increases in striatal DA content. Alternatively, baclofen exerted an acute inhibitory effect on depolarization-induced activation of tyrosine hydroxylase in the striatum (Arias-Montano et al., 1991). Depending on the localization and density of the GABA-B receptor, baclofen might exert differential effects on dopaminergic and serotonergic systems. The inability of baclofen to exert a significant effect on acute MDMA-induced 5-HT deficits is in line with studies using DA synthesis inhibitors. Depletion of intraneuronal DA using α-methyl-para-tyrosine was shown to reduce the degree of acute MDMA-induced release of DA, whereas the drug failed to block the acute release of 5-HT mediated by MDMA. On the other hand, dramatic reductions in MDMA-induced loss of 5-HT markers were observed seven days later (Brodkin et al., 1993; Schmidt et al., 1992). Thus, the antagonism by baclofen of the MDMA-induced increase in dopamine synthesis might be critical for the prevention of neurodegenerative effects, at least in the striatum.

In contrast to the striatum, MDMA produced opposite changes in DA content in the cortex (table 1). Paradoxically, baclofen pretreatment potentiated the increase in DA content elicited by MDMA, but significantly attenuated acute MDMA-induced serotonergic deficits. This observation is consistent with a study, using haloperidol, a D_2 receptor antagonist (Schmidt et al., 1992), whereby increased DA synthesis was observed following systemic administration of the drug. Likewise, the increase in cortical DA content observed in rats receiving a combination of baclofen and MDMA could be attributed to the interruption of impulse flow in A10 dopaminergic neurons caused by baclofen, consequently resulting in decreased extracellular DA and leading to reduced occupancy of presynaptic D_2 autoreceptors, hence, relieving the tonic feedback inhibitory effects on DA synthesis exerted by synthesis regulating D_2 autoreceptors. Furthermore, a recent study has shown that haloperidol treatment effectively attenuated the neurotoxic loss of 5-HT markers induced by MDMA in the forebrain regions (Colado et al., 1999d). Consistent with the present study, Colado et al. (1994) showed that chlormethiazole, a GABA mimetic, produced a modest (~30%) increase in the MDMA-induced increased striatal DA content 4 h later and a decrease in MDMA-induced 5-HT deficits 7 days later. The technique of determination of tissue monoamine content fails to differentiate between increased synthesis, or decreased degradation, or increased reuptake of the monoamines upon exposure to baclofen and MDMA combination. Therefore, the observed neuroprotective effects of baclofen in the present study presumably, occurs due to partial reduction of excessive dopaminergic transmission facilitated by MDMA in the striatum. Alternatively, baclofen induced elevation of tissue DA content in the cortex of MDMA treated rats may occur by some unknown mechanism.

We have previously hypothesized that depletion of 5-HT neuronal stores, together with excessive DA release, creates the conditions for neurodegeneration. For example, Sprague et al. (1995) demonstrated that pretreatment with 5-hydroxytryptophan (5-HTP), a precursor of 5-HT attenuated long term MDMA-induced loss of 5-HT markers. The authors hypothesized that despite MDMA's ability to promote acute release of 5-HT, increasing neuronal 5-HT content was able to counteract the neurodegenerative effects of MDMA. Likewise, activation of presynaptic GABA-B heteroreceptors might have resulted in hyperpolarization of the 5-HT terminal, which in turn might lead to a decrease in 5-HT release, thereby indirectly antagonizing MDMA's ability to deplete cortical serotonin stores. Indeed, the current result is consistent with that of Bowery et al., (1980), who showed that baclofen produced significant reductions in potassium induced 5-HT release from frontal cortical slices. The authors hypothesized that GABA-B receptors might be located presynaptically on 5-HT terminals. Taken together, the observed regional differences in DA and 5-HT content are more likely to result from regional differences in the sensitivity of dopaminergic and serotonergic transmission to baclofen. Nevertheless, the possibility exists that baclofen induced changes may occur by a hitherto unknown mechanism.

Our demonstration that stimulation of GABA-B receptors by baclofen has marked effects on monoaminergic transmission prompted us to investigate whether baclofen's neuroprotective effect is attributable to selective GABA-B receptor activation. For this purpose we used a high affinity GABA-B antagonist, SCH-50911. Previously, Erhardt et al., (1999) demonstrated that when SCH-50911 was given systemically or locally by microintophoretic techniques, an increase in firing rate and burst firing activity of

dopaminergic neurons (which represents vesicular release) was observed. The authors hypothesized that the GABA-B antagonist was blocking somatodendritic receptors on nigral dopamine neurons thereby facilitating DA release. Based on the hypothesis that MDMA exerts an inhibitory effect on striatonigral GABAergic neurons with a subsequent disinhibition of nigrostriatal doapminergic neurons (Yamamoto et al., 1995), we hypothesized that further inhibition of GABAergic transmission using SCH-50911 would exaggerate the MDMA induced depression of GABAergic transmission. This effect might be expected to translate into potentiation of the MDMA-induced neurotoxic response. Paradoxically, SCH-50911 pretreatment failed to potentiate the neurodegenerative changes elicited by MDMA. On the other hand, attenuation of the neurotoxic response was evident in the cortex. There are several possible explanations for this observation. Although SCH-50911 is known to have a DA enhancing effect, the current study is confounded by the fact that SCH-50911 also abolished MDMA-induced hyperthermia. Hence, based on the evidence that the degree of hyperthermia correlates positively with the magnitude of long-term MDMA-induced depletion of 5-HT and 5-HIAA (Broening et al., 1995; Colado et al., 1995), lowering of body temperature might have attenuated the neurotoxic response. Thus, attenuation of the MDMA-induced hyperthermic response might be beneficial even when a potentiated dopaminergic response is believed to occur.

Neuroprotection in the cortex might also be attributed to increased affinity of SCH-50911 for the GABA-B autoreceptor located on GABAergic neurons. If this was the case, then blockade of the autoreceptor might eliminate the autoreceptor mediated inhibitory effect on GABA release. This effect might then lead to increased release of

GABA and subsequent, predominant activation of presynaptic GABA-B heteroreceptors on dopaminergic terminals, which in turn might reinstate the normal negative feedback inhibitory effect on dopaminergic transmission. Indeed, Ong et al., (1998) showed that SCH-50911 increased the electrically-evoked release of [^{3}H] -GABA from rat neocortical slices, which suggests that this compound is an antagonist at GABA-B autoreceptors.

On the other hand, MDMA-induced 5-HT release onto 5-HT_{2A} receptors may shut down GABA interneurons to such an extent that an antagonist just cannot exert any further inhibitory effect on GABA neurotransmission. Despite several putative mechanisms forwarded to explain the failure of SCH-50911 to enhance MDMA-induced neurotoxicity, baclofen mediated protection against MDMA-induced neurotoxicity may also involve mechanisms independent of GABA-B receptor activation. Thus, the current study using a GABA-B receptor antagonist does not unequivocally address the role of GABA-B receptors, given the diverse pharmacological effects on distinct types of GABA-B receptors: (auto verses hetero) along with the drug's ability to block MDMA-induced hyperthermia.

In summary, the experiments outlined in this study demonstrate the ability of baclofen to attenuate both MDMA-induced hyperthermia and neurotoxicity. Furthermore, consistent with the hypothesis of dopamine involvement in MDMA-induced neurotoxicity, this raises the possibility that baclofen-mediated significant reduction of MDMA-induced increases in dopaminergic transmission might underlie the neuroprotective effects. The depressant effect of baclofen on MDMA-induced potentiation of monoaminergic transmission is likely to be involved in the underlying

mechanism of neuroprotection. Nevertheless, the importance of hypothermia in preventing the MDMA-induced long-term serotonergic deficits cannot be overlooked.

Role of the Dopamine Transporter (DAT)

The current results confirm and extend previous findings that the MDMA-induced increase in extracellular DA is an essential mediator in the long-term decrements of 5-HT markers. In the present study, we demonstrated that the DAT might be involved in these long-term deficits. Consistent with previous observations, MDMA alone produced pronounced long-term reductions in serotonergic markers, however, dopaminergic markers remained unchanged. In rats whose striatal DAT levels were dramatically reduced by AS-mediated translational arrest, a significant attenuation of the neurotoxic response was evident. Interestingly, however, AS infusion failed to prevent the hyperthermic response elicited by MDMA. Taken together, the data are supportive of a view that excessive dopaminergic transmission induced by MDMA is involved in a series of neurotoxic events that culminates in the loss of 5-HT terminal integrity. Importantly, our study also suggests that MDMA-induced impairment in DAT may not directly contribute to the hyperthermic response produced by MDMA.

The antisense technology utilized in the current study has emerged as a powerful alternative to conventional pharmacological manipulations. Antisense oligonucleotides are short stretches of synthetic, chemically modified, DNA that hybridize to the complementary mRNA sequence based on Watson and Crick's base pairing rules (Cooper et al., 1999). The resulting duplex can lead to the translational arrest of the

protein enocoded by the targeted transcript by a variety of mechanisms, including inhibition of splicing, inhibition of protein translation by disrupting ribosome assembly, and most commonly, through RNase-H recognition of RNA-DNA duplexes and subsequent selective cleavage of the RNA strand, the most widely recognized mechanism that is involved (Koller et al., 2000; Kanaya, 1995). The hallmark of antisense (AS) oligonucleotides (ODNs) is the remarkable specificity that is less likely to be achieved with pharmacological intervention.

To determine the efficacy of AS-ODNs in reducing the number of DAT sites, we performed radioligand binding studies with [^{3}H]-mazindol, a radioactive label for the DAT. In the first set of experiments we tested the efficacy of AS-I. Constant infusion of AS-I into the SNc produced a 40% reduction in labeled DAT sites, consistent with the results of Sylvia et al. (1996). Based on the study of Sylvia et al. (1996) that a 40% reduction in DAT is sufficient to modulate the amphetamine-induced locomotor response, we tested the efficacy of AS-I in attenuating the neurotoxic response elicited by MDMA. Surprisingly, no significant attenuation of MDMA-induced neurotoxicity was observed. In our later studies we realized that AS-I had only 86% homology to the DAT mRNA, therefore in the absence of 100% complementarity to the DAT mRNA, the 40% reduction in DAT sites might represent some nonspecific effects of the ODN. Thus, the incorrect AS infusion might have produced the level of translational arrest that is not sufficient to modulate the neurotoxic effects of MDMA.

In the second set of experiments we utilized an end-capped AS sequence (AS-II) (Simantov et al., 1996) directed against the exon portion of the 5' splice junction of the third transmembrane domain. This sequence produced only 40% reduction in [^{3}H]

mazindol labeled sites. MS-II had no significant effect on [^{3}H]-mazindol labeled sites. In addition, DA levels in the striatum were not altered significantly in AS-II and/or MS-II treated rats, ruling out any artifactual effects of the ODNs. Also, ODN treatment produced an apparent toxic response in a subpopulation of rats (n = 2) that resulted in death 10 days following the infusion period.

In these studies, constant infusion with AS clearly exhibited sequence dependent effects in its ability to mediate DAT translational arrest. The antisense (AS-III) that resulted in maximal reduction in DAT expression was targeted to the translational start site. Indeed, antisense sequences that are complementary to the translation initiation codon and surrounding sequences are believed to inhibit the translation of the target protein by steric blockade of ribosomal subunit binding to mRNA at the 5'-cap site (Crooke 1992). After constant infusion of AS-III, the number of [^{3}H]-mazindol binding sites was significantly reduced (~70%) in comparison to saline and MS-III treated rats. The reduced efficiency of AS-II in mediating substantial DAT knockdown (KD) could be attributed either to reduced cellular uptake of the ODN or increased degradation of the ODN by the nucleases in the brain. AS-II was only endcapped and therefore a complete phosphorothioation might have imparted increased nuclease resistance (Bernard et al., (1998) and a higher percentage of knockdown. The above explanations are speculative in nature, however, and therefore need further investigation.

The dosage and continuous infusion protocol was based on previous studies demonstrating that short-term microinjections into the substantia nigra (SNc) and lower doses of ODNs failed to produce the desired levels of DAT reduction (Sylvia et al., 1996). In this context, several studies have highlighted the fact that the turnover rate of

the protein is a critical determinant of attaining maximal reduction of the target. Thus, based on studies demonstrating that DAT-turnover time is approximately 6.3 days (Fleckenstein et al., 1996), we followed the protocol that involved constant infusion for 1 week. Furthermore, the rationale behind using site-directed knockdown was to ascertain whether temporary downregulation of DAT content was sufficient to counteract the neurotoxic effects of MDMA. Indeed, substantial DAT reduction prior to MDMA administration produced a neuroprotective effect that was confined to the striatum. These results confirm the feasibility of the AS technique, which provides the dual advantage of target specificity coupled to anatomical selectivity.

There is considerable evidence demonstrating the involvement of nonvesicular, calcium-independent release of DA, presumably via a carrier-mediated "exchange-diffusion" process in the psychomotor stimulant induced release of DA (Fischer and Cho 1979). Pretreatment with DAT inhibitors was previously shown to attenuate increased efflux of DA in the striatum following local (Nash and Brodkin 1991) or systemic administration (Shankaran et al., 1999) of MDMA. For example, Shankaran et al. (1999) using microdialysis studies, demonstrated that both sustained MDMA-induced increases in the extracellular concentration of ROS and long-term depletion of 5-HT in the striatum were attenuated by mazindol pretreatment. These results point to a role for the DAT in mediating acute release of DA and consequent formation of ROS, a potential mechanism for long term loss of 5-HT terminals in the striatum. In a similar fashion, pretreatment with mazindol or GBR 12909 was found to significantly inhibit the increases in extracellular DA produced by local infusion of MDMA into the striatum. The authors concluded that, similar to amphetamine, mazindol is blocking the binding of MDMA to

the DAT in the axon terminal, thereby preventing the release of DA believed to be mediated by the DAT. Despite mazindol's inhibitory effect on the DAT, mazindol is also a potent inhibitor of norepinephrine uptake. This problem is, however, circumvented in the current study whereby the DAT-AS produces a selective knockdown of the DAT.

DAT-AS-III treatment led to almost complete reversal of the long-term reductions in striatal 5-HT markers, whereas MS-III treatment failed to alter the MDMA-induced neurotoxic effects. Taken together, the results of the current work, in conjunction with the above mentioned studies, are supportive of the hypothesis that excessive DA release, mediated through the DAT, serves as an intermediate for the formation of neurotoxic metabolites that might ultimately lead to 5-HT terminal loss.

The significance of nonvesicular DA in MDMA-induced long-term deficits is further illustrated by the following studies. Johnson et al., (1991) demonstrated that a potent nonvesicular releaser of 5-HT, 5-methoxy-6-methyl-2-aminoindan (MMAI), produced marked long term reduction in 5-HT markers only when combined with amphetamine, a non vesicular DA releaser, while neither drug alone produced neurotoxicity. Conversely, increased DA synthesis by amphetamine and concomitant activation of the 5-HT_2 receptor by R-DOI was not sufficient to produce long term reductions in 5-HT markers in the striatum. Those studies hypothesized that 5-HT depletion from the 5-HT presynaptic terminal somehow renders the 5-HT terminal susceptible to the neurotoxic effects of MDMA. Similarly, in a recent report Aguirre et al., (1998) demonstrated that administration of MDMA to rat pups at postnatal day 21 (PND 21), where the rat is believed to have a poorly developed dopaminergic system, failed to produce neurotoxicity. Pretreatment with L-DOPA followed by MDMA,

however, did produce persistent reduction of 5-HT markers in the hippocampus and frontal cortex. Conversely, 6-OHDA lesioned rats failed to display long lasting reductions in 5-HT markers following MDMA, but administration of L-DOPA reinstated MDMA's neurotoxic effects. These results seem to confirm the obligatory requirement of a fully functional dopamine system in order to sustain 5-HT neuronal injury.

In this context, numerous studies have implicated DA as a potential neurotoxic agent. For example, DA can undergo enzymatic oxidation via monoamine oxidase-B (MAO-B) to generate H_2O_2 with subsequent formation of superoxide or hydroxy radicals (Halliwell 1992). On the other hand, DA can undergo autooxidation to the corresponding chemically reactive quinones (Graham et al., 1978). Thus, DA might gain access to the interior of the 5-HT terminal and facilitate the loss of 5-HT terminal integrity through an oxidative stress mechanism. Indeed, Sprague et al., (1995) demonstrated that L-deprenyl blocked MDMA-induced formation of thiobarbituric acid (TBA) reactive substances, considered to be an indirect marker of membrane lipid peroxidation. Deprenyl also prevented subsequent loss of 5-HT markers in the striatum, hence implicating oxidative products of DA in the neurotoxicity process. Another mechanism by which DA quinones could facilitate MDMA-induced neurotoxicity includes inactivation of TPH through covalent modification of critical cysteinyl residues, leading to direct loss of the enzyme's catalytic effect. This view is consistent with a recent in vitro study (Kuhn and Arthur, 1998) demonstrating the detrimental effects of DA in mediating the loss of TPH activity. The authors hypothesized that TPH-quinoprotein could be formed in vivo under conditions of elevated extracellular DA levels or elevated DA synthesis. Additional support for the ROS mediated inactivation of TPH following MDMA administration

arises from studies showing that MDMA-induced depletion of 5-HT and 5-HIAA and loss of TPH were attenuated by reducing agents such as dithiothreitol plus Fe^{2+} (Stone et al., 1989), and L-cysteine (Schmidt et al., 1990). Collectively, DA might serve as an important source of ROS that could lead to oxidative damage of 5-HT terminals.

A neurotoxic dosing regimen of MDMA generally produces hyperthermia in experimental animals. Indeed, the results of the current studies illustrate the hyperthermic effects of MDMA. Interestingly, rats that underwent DAT knockdown via AS infusion failed to show an attenuated hyperthermic response following MDMA. Previous studies have shown that attenuation of MDMA-induced hyperthermia or pharmacological manipulations that induce hypothermia in the presence of MDMA diminish the neurotoxic effects of MDMA (Colado et al., 1997, Colado et al., 1999). In the present studies, neuroprotection was afforded without altering the drug-induced hyperthermic response. Indeed, our results are consistent with those of Shankaran et al. (1999), who showed that pharmacological blockade of the DAT using mazindol prior to MDMA had no significant effect on MDMA elevation of body temperature. Hydroxy radical formation and subsequent loss of 5-HT levels in the striatum were, however, attenuated. In a similar fashion, Aguirre et al., (1998) recently demonstrated that even in the presence of hyperthermia, rat pups were not vulnerable to the neurotoxic effects of MDMA, emphasizing that DA rather than hyperthermia is an essential factor for the expression of neurotoxicity. In an analogous fashion, although fluoxetine prevented the long-term loss of 5-HT markers, the drug failed to reverse the hyperthermia produced by MDMA. Taken together, hyperthermia per se is not the sole contributing factor in MDMA-induced neurotoxicity. These findings are consistent with the hypothesis that

hyperthermia alone does not predispose 5-HT neurons to undergo neuronal degeneration, but rather, that hyperthermia may enhance the toxic process under conditions when the antioxidant status of the 5-HT neuron is overwhelmed or exhausted.

In summary, the present studies suggest that the DAT is involved in MDMA-induced neurotoxicity. When expression of the DAT was reduced by AS-ODN, attenuation of MDMA-induced long-term reductions in serotonergic markers was observed. Interestingly, knockdown of the DAT failed to attenuate MDMA-induced hyperthermia. The current study is consistent with the hypothesis that DAT-induced DA release is a critical mediator of neurotoxicity and provides further evidence for the involvement of the DA uptake carrier. Moreover, the occurrence of neuroprotection, in spite of MDMA-induced hyperthermia, argues against a role for hyperthermia as a critical determinant of neurotoxicity. Regardless of the mechanism of action, hyperthermia and neurotoxicity are not inextricably linked. The current study also underscores the feasibility of using site-directed, temporally-limited, knockdown of the target protein by constant AS infusion to facilitate the delineation of the role of a particular protein in a neurochemical pathway.

A Role for COX-2 Activation

As we and others have shown, that MDMA-induced neurotoxicity involves DA as well as mechanisms associated with oxidative stress. Our data indicates that COX-2 mediated reactive products might be involved in the neurodegenerative effects of MDMA in a region specific manner.

Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid (AA) to prostaglandin (PG) and thromboxanes. COX-1 is constitutively expressed in most tissues, however, COX-2 is expressed upon induction by several pro-inflammatory processes including inflammation, fever, and neurodegenerative events. In fact, ROS are believed to be produced by the peroxidase step of the COX reaction in the presence of a cosubstrate such as DA, during which PGG_2 is converted to PGH_2 (Hastings 1995, Mattamal et al., 1995; Armstead et al., 1988; Tsai et al., 1994). Additionally, increased COX-2 expression is known to occur during seizures (Adams et al., 1996), and ischemia (Nogawa et al., 1997). Therefore, based on compelling evidence that COX-2 can exert a regulatory effect both on hyperthermia and neuronal injury, we explored the involvement of COX-2 activation in MDMA-induced hyperthermia and neurotoxicity.

Based on numerous reports supporting a role for ROS in the neurotoxic response elicited by MDMA, we studied the impact of blockade of one such pathway, namely the contribution of COX-2 mediated generation of ROS in the long term reductions of 5-HT markers in the striatum, hippocampus, and frontal cortex in response to MDMA exposure. The results of the current study support a role for ROS generation in MDMA-induced neurotoxicity, involving regional differences in the sensitivity to prior COX-2 inhibition. SC-236 (4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]-benz-ene-sulfonamide) a selective COX-2 inhibitor, afforded protection against some of the neurotoxic effects of MDMA in the cortex, but not in the striatum or hippocampus. This finding demonstrates regional differences in the susceptibility to COX-2 mediated ROS and subsequent loss of 5-HT terminal integrity. Despite the failure of SC-236 to completely attenuate MDMA-induced hyperthermia, SC-236 pretreatment did produce a

significant reduction in body temperature for the first 4 hours, in comparison to MDMA treated rats. Despite significant blockade of MDMA-induced hyperthermia, neuroprotection was evident only in the cortex. This finding might indicate that COX-2 mediated free radical generation is predominant only in certain brain regions. The data provide further evidence that MDMA can disrupt thermoregulatory mechanisms independent of drug-induced neurotoxicity. The dichotomy presented by SC-236 induced reduction in the body temperature of MDMA treated rats, without alteration of neurotoxicity in the striatum and hippocampus, argues again against a role for hyperthermia as the sole mediator in the neurotoxic effects elicited by MDMA.

Several mechanisms could be advanced to explain COX-2 mediated neuronal injury. Events that favor COX-2 activation have been previously shown to be crucial factors for the expression of MDMA-induced 5-HT terminal loss. For example, previous reports showed that 1) DA can be oxidized via prostaglandin H synthase (COX), therefore leading to increased formation of protein cysteinyl-DA, an oxidative metabolite that participates in cell death (Mattamal, 1995; Hastings 1995). In fact, DA-mediated oxidative products have been implicated in the long-term degenerative effects of MDMA (Sprague et al., 1995). 2) Mitochondrial dysfunction due to cytochrome oxidase inhibition can result in ROS formation through activation of cyclooxygenase (Gunasekar et al., 1998). Consistent with that report, MDMA was shown to produce a transient decrease in cytochrome oxidase activity localized to DA rich regions such as striatum and nucleus accumbens, consistent with the involvement of quinones or other free radicals in long term neurotoxicity (Burrows et al., 2000). 3) Release of nitric oxide (NO) and subsequent activation of nitric oxide pathways is also known to increase COX-2

expression (Nogawa, 1998). In light of this evidence, MDMA-induced neurotoxicity was also shown to be attenuated by NOS inhibitors (Zheng and Laverty, 1998). Taken together, our data suggests that MDMA-induced neurotoxicity may involve neurotoxic events that promote COX-2 activation at least, in the cortex. Thus, modulation of COX-2 activity might lead to significant prevention of MDMA-induced neurotoxicity in certain regions.

Previously, other COX-2 selective inhibitors have been shown to be neuroprotective by virtue of their ability to block ROS generation that led to loss of neuronal morphology (Nogawa et al., 1998). Thus, we propose that SC-236 affords protection against MDMA damage by a similar mechanism, although we do not have direct evidence in support of that contention. Nevertheless, based on the fact that MDMA produces acute increases in hydroxy radicals (Shankaran et al., 1999) by a DA-dependent mechanism, coupled with the fact that peroxides act as important co-substrates in COX-2 mediated oxidative formation of quinones, COX-2 inhibition might attenuate free radical generation, particularly in regions such as the cortex where COX-2 might be activated upon generation of pro-oxidant species. The current results are preliminary in nature, because in the absence of a dose-response curve for SC-236, we are unable to dissect the influence of dosing regimen or pharmacokinetic parameters in the mechanism of action. Thus, the lack of complete neuroprotection observed with the COX-2 inhibitor stems from the lack of an effective drug concentration at the target site. This possibility is rendered more likely by the fact that SC-236 has extremely poor water solubility. Irrespective of these caveats the present studies demonstrate that COX-2 might be partly involved in the neurotoxic response elicited by MDMA.

Pharmacological agents or environmental conditions that modulate MDMA-induced hyperthermia are also known to be neuroprotective (Colado et al., 1997; Malberg et al., 1995). The present study demonstrates that the MDMA-induced pyretic response may be partly dependent on COX-2 activation, because only a modest degree of attenuation of MDMA-induced hyperthermia (<5 hours) was observed. In addition, the absence of antagonistic effects of SC-236 on MDMA-induced hyperthermia at later time points suggests that distinct phases of hyperthermia might be mediated by differerent mechanisms. Owing to the complexity of the hyperthermic response elicited by MDMA, rather than a single mechanism causing hyperthermia, a myriad of MDMA-induced neurotoxic events probably culminate in the disruption of thermoregulatory mechanisms. The current results suggest that hyperthermia may exaggerate the neurotoxic response elicited by MDMA, however, hyperthermia does not play a causal role in the 5-HT neuronal loss elicited by MDMA.

In summary, our data demonstrate that SC-236 exhibits regional differences in its ability to attenuate the MDMA-induced neurotoxic response. Therefore, COX-2 activation might be partially responsible for the long-term reductions in 5-HT markers produced by MDMA, at least in some brain regions. The failure of SC-236 to reverse completely the MDMA-induced hyperthermia suggests that, COX-2 activation is not the sole contributor in MDMA-induced hyperthermia. Furthermore, the results suggest that in the presence of agents that reduce the extent of free radical generation, hyperthermia might only play a minor role in the development of MDMA neurotoxicity.

Summary and Conclusions

The major goal of my project was to understand better the neurochemical mechanisms underlying MDMA-induced 5-HT terminal degeneration. Based on our experimental results, along with the existing literature, we propose that the local microenvironment and neural substrate availability appear to be key determinants for the regional differences in the vulnerability of 5-HT neurons to the neurotoxic effects of MDMA. For example, at the level of the striatum it is possible that the DAT might exert a more predominant role, unlike in cortex, where impulse dependent monoaminergic firing might be more critical. We suggest the following models for the long-term loss of 5-HT terminal degeneration in the striatum and the cortex:

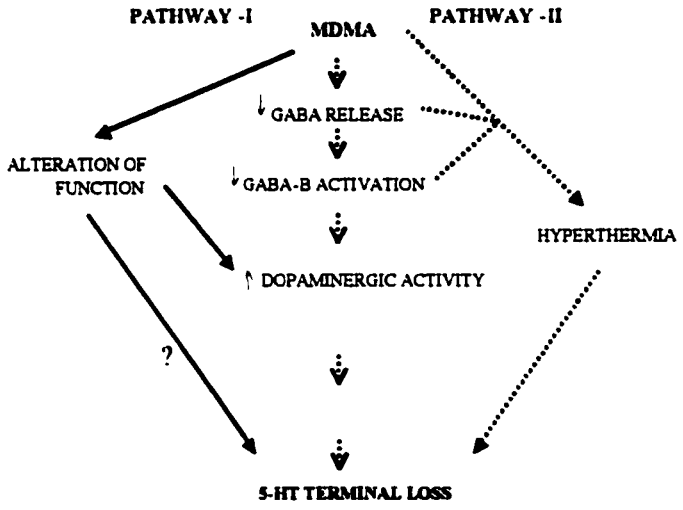

Figure 17. Putative model for MDMA-induced 5-HT neurotoxicity in the striatum

Figure 17. is a schematic representation of the proposed model that may involve the occurrence of the following sequence of events in the striatum. Apparently, MDMA induced excessive dopaminergic transmission may occur via two parallel pathways namely, non-vesicular carrier mediated (pathway-I: solid arrows; major pathway) and impulse dependent mechanisms (pathway-II: dotted arrows; minor pathway). The exact contribution of these individual pathways to the MDMA-induced neurotoxicity is currently unclear. But, reduction of DAT levels and enhancement of GABAergic neurotransmission prior to MDMA appears to protect the 5-HT neurons from the neurodegenerative effects of MDMA.

Pathway-I:

1. MDMA administration causes reversal of the DAT, and thereby indirectly enhancing the synaptic availability of DA.
2. The resulting excessive synaptic DA presumably is taken up into the 5-HT terminals thereby leading to loss of 5-HT terminal integrity via a dopamine dependent process.
3. The DAT apparently is a key intermediate in the neurochemical mechanisms underlying MDMA-induced neurodegeneration. It is unlikely that excessive activation of the DAT is a contributing factor in MDMA-induced hyperthermia. Our results are supportive of a hypothesis that DAT activation and hyperthermia are independent events.

Taken together, our results demonstrate the obligatory role of the DAT in the MDMA-induced serotonergic terminal degeneration. Nevertheless, hyperthermia is not sufficient for the development of MDMA-induced neurotoxicity.

Pathway II:

1. MDMA may induce an acute reduction in GABA levels through suppression of the striatonigral GABAergic system via 5-HT $_{2A}$ receptors.
2. Reduction in the synaptic GABA levels may lead to a perturbation of the normal negative feedback inhibitory effects mediated by GABA-B receptors activation on striatal dopaminergic neurons.
3. This reduced activation of the GABA-B receptor might then lead to potentiate DA synthesis and release.
4. The resulting excessive DA neurotransmission might contribute to long-term reductions of 5-HT markers.
5. MDMA-induced alterations in GABAergic neurotransmission might mediate, at least in part, the hyperthermic response elicited by MDMA.

Collectively, our data are consistent with the hypothesis that reduced GABAergic tone might indirectly lead to the disinhibition of the nigrostriatal dopaminergic sytem, resulting in persistent reductions in the 5-HT markers, at least in the striatum. Alternatively, it is also possible that attenuation of MDMA-induced hyperthermia by GABAergic agents might have ameliorated the extent of neuronal degeneration induced by MDMA. The current study highlights the neuroprotective effects associated with attenuating dopaminergic activity and hypothermia on MDMA-induced neurotoxicity, however, the exact contribution of hypothermia to the neuroprotective effects of GABAergic agents remains unclear and, therefore, warrants further investigations.

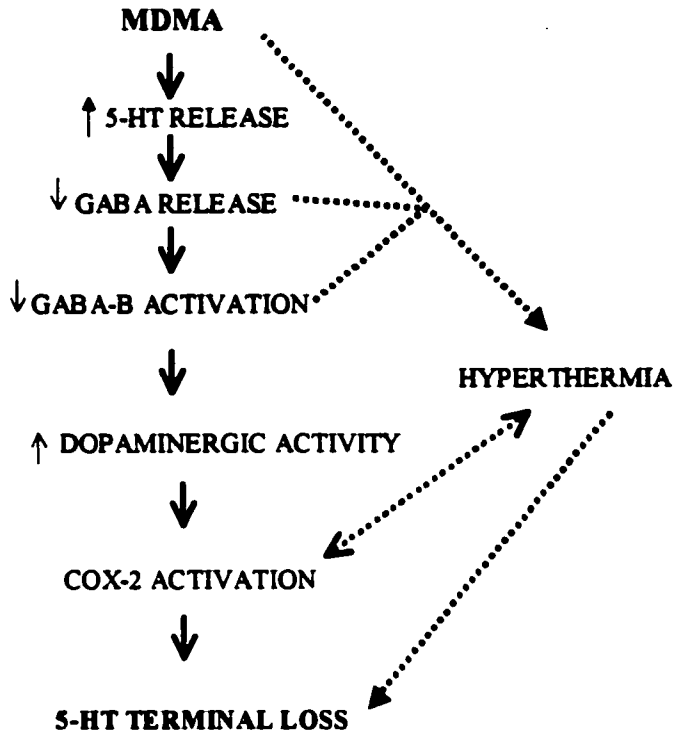

Figure 18. Putative model for MDMA-induced 5-HT neurotoxicity in the cortex

Based on our experimental results, we suggest the occurrence of the following sequence of events that might contribute to 5-HT neurotoxicity in the cortex (figure 18).

1. MDMA induces a pronounced acute release of 5-HT. The acute release of 5-HT might be partially attributed to decreased activation of the GABA-B receptors.
2. The reduced synaptic concentrations of GABA together with reduced activation of GABA-B receptors located on the dopaminergic neurons might relieve the tonic inhibitory input exerted by GABA-B receptors on the dopaminergic neurons thereby leading to excessive dopaminergic activity.

3. The excessively released DA, presumably acting as a cosubstrate for COX-2, might have contribute to loss of 5-HT terminals through a prostaglandin or ROS-dependent process.

4. The inherent hypothermic property of GABAergic agents might also contribute to the overall neuroprotective effects associated with GABAergic agents such as GVG and baclofen. The exact relationship between hypothermia and neuroprotection in the mechanisms underlying GABAergic agents remains to be examined.

Taken together, our results indicate that impairments in GABAergic neurotransmission might also be partially responsible for long-term reductions of 5-HT markers in the cortex.

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VITA

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Arthi Kanthasamy was born on February 17, 1970 to Dr. Puddupalayam. kumaraswamy.Rangiah and Victoria Gunaseeli Rangiah in Madurai, India. After graduating from St Avila, high school (Coimbatore, India) in 1987, she attended PSG College of Sciences where she studied biochemistry for 3 years. She received her B.S. in biochemistry in 1990, graduating third in the class. She went on to do her masters in biochemistry in 1990, in the same institution. She was happily married to Dr. Anumantha G. Kanthasamy, Ph.D on May 26, 1991. The following year she started her family with the birth of her son Kavin on December 1, 1992. She worked as a technician for Dr David E. Nichols from 1992-1994. In 1995, she left for California where she studied at UCI for a short while, prior to her departure to Purdue University.

In January, 1996, she began her graduate studies at Purdue University, Department of Medicinal Chemistry and Molecular Pharmacology. Under the guidance of her major professor, Dr. David E. Nichols, she enjoyed working on elucidating the role of excessive dopaminergic transmission in MDMA-induced neurotoxicity. She was supported for the entire period from DA-04758 form the National Institute on Drug Abuse. During her graduate school training, Arthi was elected to the society for Neuroscience, and won the 1998 Purdue University Neuroscience outstanding presentation award. She graduated with a Ph.D. degree in May of 2001 and began her post-doctoral training with Dr Richard Martin (Department of Biomedical Sciences) at Iowa State University (Ames, IA).